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EDITED BY
SIMON FLEXNER, M.D. PEYTON ROUS, M.D.

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REACTIONS OF RABBITS TO NON-HEMOLYTIC STREPTOCOCCI

III. A STUDY OF MODES OF SENSITIZATION

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(Received for publication, April 3, 1930)

In previous papers (1, 2) we have described the various types of tissue response of rabbits to inoculation with non-hemolytic streptococci. It was shown that reactions following reinfection were conditioned by the route of the original inoculation: if it had been into the tissues—especially into the skin—the subsequent reaction was that of hypersensitiveness, or hyperergy; if into the blood stream, it was that of immunity. For the sake of completeness two other types of reaction were recorded: that of normal animals, which in the majority of instances was followed by a secondary reaction, and that of cachectic rabbits, in which inoculation resulted in a soft, pale lesion smaller than that of normal animals, and not followed by a secondary reaction.

Variability in the response of apparently normal rabbits to inoculation with streptococci is well recognized; and because of this some investigators feel that this species is unsuitable for the study of experimental streptococcus infections. Under ordinary experimental conditions this factor necessitates the introduction of a sufficient number of animals into each experiment to permit of conclusions on a statistical basis. While such a basis is always requisite in biological research the advantages of having to depend upon it as little as possible are obvious.

During our investigations the desirability became more and more evident of ascertaining the best method of rendering a large majority of rabbits satisfactorily hypersensitive to non-hemolytic streptococci in the shortest possible time. This communication gives the details of some of the experiments planned with such an end in view.

EXPERIMENTAL

Both green-producing and indifferent strains of non-hemolytic streptococci of previously demonstrated sensitizing capacity have been used. From an original stock culture, preserved in the frozen and dried state, sub-stocks in rabbits' blood broth were prepared every 6 to 10 weeks. As the work progressed it seemed that old stock cultures in blood broth gradually lost their sensitizing capacity; more recently, therefore, the blood broth stocks have been renewed from the dried culture every 4 to 6 weeks. The inoculum for the animals was a culture grown for from 18 to 24 hours in 2 per cent rabbits' blood broth to which 0.05 per cent dextrose was added. Both Douglas tryptic broth and beef infusion broth have been used. If the sediment of more than 0.1 cc. of whole culture was to be injected in one place the culture was centrifuged and sufficient of the supernatant fluid was discarded so that 0.1 cc. of the residue represented the amount of growth selected for injection; 0.1 cc. of whole culture was injected if that was the quantity desired: and in the case of the smaller inocula the culture was diluted with broth or Ringer's solution so that 0.05 cc. represented the amount injected. It was found that the intracutaneous injection of 0.05 cc. of some samples of broth gave early evanescent, edematous lesions in some highly sensitized rabbits and occasionally in normal controls; but these lesions never became indurated as did those following injection of culture. Dilution of the culture with Ringer's solution did not give rise to these false reactions; hence only this diluent has been employed recently. All skin lesions were measured daily until marked diminution in size was evident, and the volumes were estimated as previously described (1). The ophthalmic and lethal tests were carried out as previously noted (1); the dose of culture employed for the lethal test was the centrifugate of 4 cc. of blood broth culture per kilogram of rabbit.

. To obtain as nearly uniform conditions as possible animals of corresponding weight and color were distributed among the different groups. During the past year we have found that Chinchillas and New Zealand Reds have more consistently become hypersensitive after intradermal inoculation and, on the whole, have had more suitable skins; hence these varieties have been used when obtainable.

Attempts were first made to determine which was the most efficacious in sensitizing animals: one, two or four intracutaneous injections, each with the sediment of 5 cc. of culture. Lesions produced with such large doses frequently broke down and discharged their contents within a few days. Another disadvantage in injecting such large doses was the failure, at times, of the resulting lesions to show clear-cut secondary reactions, whereas those produced by 10^{-1} cc. or 10^{-2} cc. in the same animals gave distinct evidence of such reactions.

With equivocal secondary reactions in animals treated with large inocula alone it was necessary to resort to ophthalmic and lethal tests to ascertain the state of reactivity. In two or three different experiments the results were so variable that no satisfactory conclusions could be drawn from the exclusive use of these large inocula. It was then observed that rabbits became increasingly sensitive following initial intracutaneous inoculation with the sediment of 5 cc., 1 cc., 10^{-1} cc., and 10^{-2} cc. of blood broth culture if they subsequently received repeated injections of 10^{-1} cc. and 10^{-2} cc. at 10-day intervals. In carrying out this procedure so many areas of skin were inoculated that little remained available for subsequent observations; hence it became desirable to devise other modes of sensitization in which less cutaneous damage was inflicted.

Experiment 1.—Eighteen rabbits were each inoculated intracutaneously in five areas with the sediment of Culture V110A/0/13, as follows: 5 cc., 5 cc., 10^{-1} cc., 10^{-2} cc., 10^{-4} cc. They were then divided into three equal groups, A, B and C. On the 8th day each animal of Group A received a subcutaneous focus of 5 cc. of agar infected with the sediment of 5 cc. of culture. The animals of Group B were inoculated intramuscularly with 10^{-1} cc. and 10^{-2} cc. from a similar culture every 5 days from the 5th to the 40th day, and those of Group C with the same doses intracutaneously. The degree of skin reactivity of Group A animals was tested with 10^{-4} cc. of culture on the 1st, 15th, 25th, 35th and 40th days, and that of the animals in the other two groups on the 1st, 10th, 20th, 30th and 40th days. The ophthalmic test was applied to the right eye of all animals on the 13th day and to the left on the 43rd day. During this interval one rabbit from each Group B and C had died. On the 48th day all of the animals except two of Group A received a subcutaneous focus of 5 cc. of agar infected with sediment from 5 cc. of culture of *Streptococcus* V110A. Six fresh rabbits comprising Group D received similarly infected agar foci. All animals were then allowed to go untreated until the 101st day, an interval of 53 days, when their right eyes were retested in the usual manner and their skin reactivity to inoculation with 10^{-4} cc. of homologous culture was measured. The comparative intensity of the cutaneous reactions to 10^{-4} cc. of culture is shown in Chart 1, and that of the ophthalmic reactions is shown in Table I.

The results clearly indicate that after the animals had been rendered hypersensitive by means of the initial skin inoculation the condition was satisfactorily maintained when an infected focus was present. At that time, however, we did not appreciate the influence of repeated intracutaneous test inoculations with 10^{-4} cc. of culture in maintaining

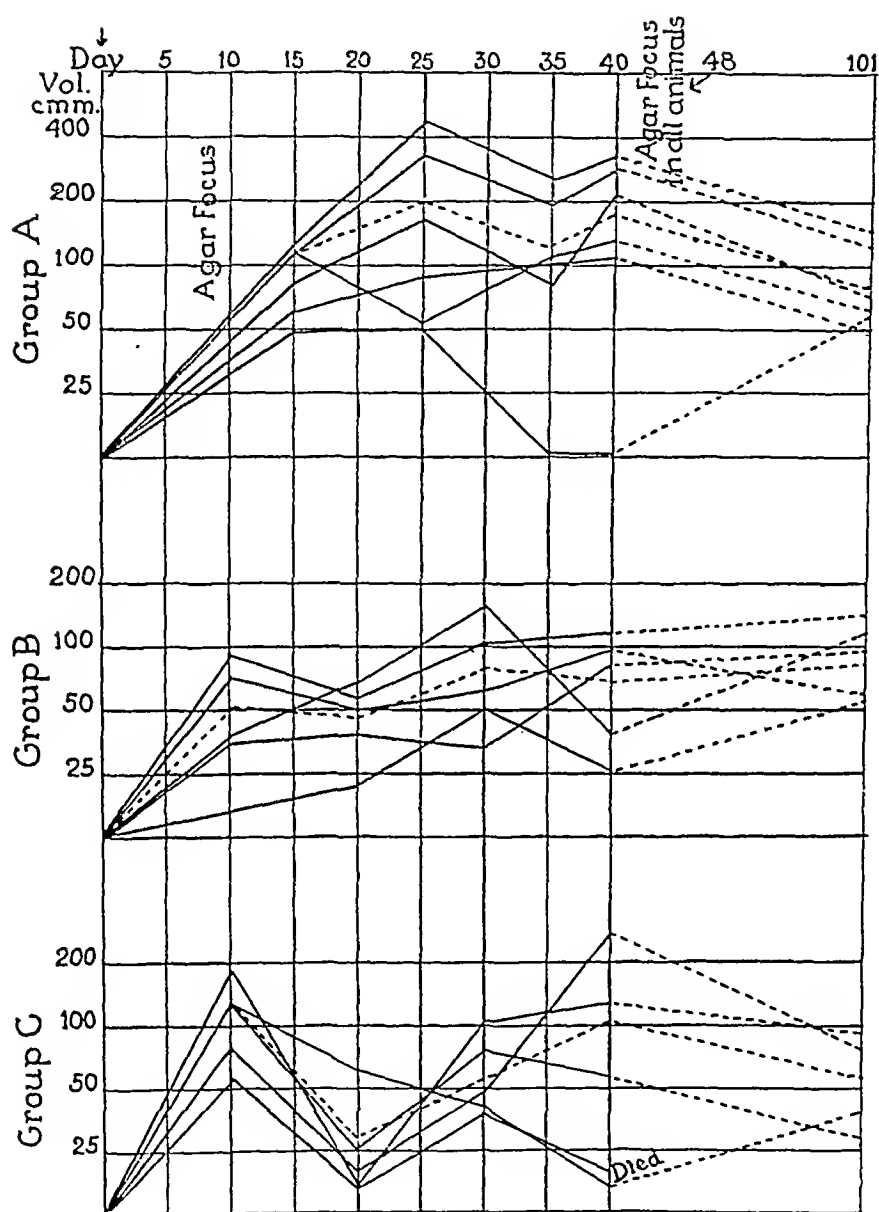


CHART 1. Comparative intensity of cutaneous reaction in rabbits sensitized in different manners.

the hypersensitive state (see Experiment 2). Yet the animals of Groups B and C also received similar small intracutaneous inoculations repeatedly at 10-day intervals concomitantly with the course of

10^{-1} cc. and 10^{-2} cc. doses, without developing a comparable degree of hypersensitiveness. Indeed, it seems probable from the appearance of the curves of Group C that the repeated intracutaneous inoculation with these larger doses may have resulted in a temporary diminution of sensitiveness at about the 20th day. This opinion was confirmed by a parallel diminution in the size of the cutaneous lesions resulting from inoculations with 10^{-1} cc. and 10^{-2} cc. of culture. Other experiments have shown a similar wave-like variation in intensity of reactions in animals receiving repeated intradermal inoculations of large doses of *Streptococcus viridans*.

In this experiment the skin lesions resulting from 10^{-1} cc. inocula were usually as large after the third and subsequent inoculations as were those produced by the sediment from 5 cc. at the time of the original inoculation. It is possible that these large cutaneous foci containing much necrotic tissue and many bacteria may have had less sensitizing effect than the smaller areas produced by 10^{-4} cc. of culture. This possibility is rendered more probable from comparative consideration of Group B. These animals, although receiving the major portion of the inocula into their lumbar muscles, always showed cutaneous lesions at the site of the needle punctures; lesions that were often of the same size as followed intracutaneous inoculation with 10^{-4} cc. of culture. They, therefore, were under the sensitizing influence of two small intracutaneous inocula every 5 days and of three similar additional ones every 10 days. Under these combined muscular and cutaneous lesions, however, the animals seemed to remain at a constantly lower level of sensitivity than existed in Group A, where the repeated sensitizing cutaneous stimuli were very small.

At the end of 6 weeks the average cutaneous and corneal sensitiveness of Group A was distinctly greater than at the end of the 2nd week, and also higher than that of Groups B and C, in which it was relatively the same as in the 2nd week. While on the 13th day none of the Group C animals gave a positive ophthalmic reaction, one did so by the 7th week (see Table I).

The effect of an infected agar focus implanted on the 48th day was to maintain or increase the corneal sensitiveness of nearly all the animals by the 101st day, even though the cutaneous reactivity of Groups A and C had fallen off to a certain degree. It is true that the

TABLE I

Comparative Intensity of Ophthalmic Reactions in Rabbits Sensitized in Different Manners

Group	Rabbit No.	13th day Rt. eye				43rd day Left eye				101st day Rt. eye			
		Degree of reaction	Days between inoculation and appearance of:			Degree of reaction	Days between inoculation and appearance of:			Degree of reaction	Days between inoculation and appearance of:		
			Congestion	Veil	Pannus		Congestion	Veil	Pannus not observed		Congestion	Veil	Pannus
A	R832	+	3	3	5	++++	1	1		++++	1	1	2
	R833	+	2	3	7	++++	1	1		+++++	1	1	2
	R834	—	—	—	—	—	—	—		±	1	1	0
	R835	++	1	2	5	++++	1	1		++++	1	1	3
	R836	±	3	4	—	+	2	3		++++	1	2	5
	R837	?	2	—	—	++++	1	1		++++	1	1	2
B	R838	+	+	1	—	+	2	2		—	—	—	—
	R839	—	—	—	—	—	—	—		++++	1	1	3
	R840	—	—	—	—	—	—	—		++++	1	2	5
	R841	+	1	1	5	++	1	2		++++	1	1	6
	R843	++	1	1	5	±	1	2		++++	1	1	3
C	R844	—	—	—	—	++	2	2		++	1	1	6
	R845	—	—	—	—	—	—	—		++++	1	1	5
	R847	—	—	—	—	—	—	—		±	1	1	0
	R848	—	—	—	—	—	—	—		†			
	R849	—	—	—	—	—	—	—		++	1	1	5
D	R878									—	—	—	—
	R879									—	—	—	—
	R880									++	7	7	9
	R881									±	8	8	0
	R882									—	—	—	—
	R883									++	4	4	6
E	Controls												
	R897									—	—	—	—
	R898									+	10	10	11
	R899									++	10	10	12
	R900									—	—	—	—

† Animal died.

+ = negative reaction.

± to ++++ = varying intensity of reaction.

right eye, which had been inoculated on the 13th day, was the one tested on the 101st day and the effect of this first inoculation must be considered. But among eight of the surviving animals in which the first inoculation of the right eye did not result in keratitis, the second—on the 101st day—was followed by distinct and intense reactions (see Table I).

These reactions, moreover, appeared promptly as compared with those of Groups D and E. Group D animals had been sensitized 53 days previously by an infected subcutaneous agar focus, without any preliminary cutaneous inoculations. Only one-half of this group developed positive ophthalmic reactions at all, and these were all weaker than in the first three groups, and were also slower in appearing. The members of Group E were introduced for the first time at the end of the experiment, to serve as controls of the activity of the culture at that time; like the animals of the other groups they received intracutaneously 10^{-1} cc., 10^{-2} cc., and 10^{-4} cc. of culture on the day their corneae were inoculated. Two developed secondary cutaneous reactions and shortly afterwards showed weak but distinct ophthalmic reactions. These two animals illustrate well that for the development of keratitis a state of hypersensitiveness is necessary; for even though their eyes were inoculated early, they showed corneal lesions only after the development of this state as indicated by the secondary reactions. The five groups of animals show clearly, as judged by their ophthalmic reactions, how variations in the degree of hypersensitiveness may be conditioned by differences in the method of previous inoculation.

Experiment 1 indicated that in most rabbits a high degree of hypersensitiveness could be obtained in 35 to 40 days by the following method: initial inoculation of several large doses of green streptococci, followed by the introduction of a single infected agar focus, and thereafter by repeated intracutaneous inoculations with 10^{-4} cc. of culture at 10-day intervals. In two subsequent experiments carried out in almost the same manner, but without skin testing every 10 days, such a satisfactory degree of hypersensitiveness was not obtained. In fact the proportion of highly sensitive animals was very disappointingly small. The only differences between the two series of experiments consisted in the absence of small intracutaneous

inoculations every 10 days in the second, and in the seasons of the year in which the two were carried out. Variations in sensitizing capacity of culture must also be considered.

Experiment 2 was therefore planned to determine whether one infected subcutaneous agar focus, or multiple intracutaneous foci from repeated single inoculations with 10^{-4} cc. of culture every 10 days, or a combination of the two, was most effective in maintaining the hypersensitive state.

Experiment 2.—Each of 64 rabbits was inoculated intracutaneously with the sediment of 5 cc., 5 cc., 10^{-1} cc., and 10^{-4} cc. of blood broth Culture V110A/0/6. On the 8th day they were divided into four groups. Into each animal of Group A (27 rabbits) and Group B (25 rabbits) was placed a subcutaneous focus of 5 cc. of melted agar inoculated with the sediment of 5 cc. of 24-hour blood broth culture. Groups C and D, containing 6 rabbits each, received no agar foci. Groups A and B were larger because it was hoped by production of agar foci to obtain many highly sensitive animals for a subsequent experiment. On the 11th day the right cornea of each rabbit was inoculated in the usual manner. The number and percentage of positive ophthalmic reactions is indicated in Table II. The proportion of animals in Groups A, B, and D, which developed secondary reactions was approximately the same; but Groups A and B had a much higher proportion of positive ophthalmic reactions than did Groups C and D. It seemed, therefore, that the implanting of agar foci at the time when the animals were becoming hypersensitive increased the sensitiveness of the eyes; this is a confirmation of similar observations in Experiment 1. The animals of Groups A and C were inoculated intracutaneously with 10^{-4} cc. of homologous culture on the 11th, 21st and 31st days. On the 42nd day the left eye of all the rabbits was tested, and all animals received 10^{-4} cc. of culture intracutaneously. The volumes of the resulting skin lesions were computed from daily measurements; the average for each group is given in Table II. Five days later the cutaneous reactivity of all the rabbits was again tested with 10^{-4} cc. of each of two cultures, one of which had been grown from an old blood broth stock preserved in the ice-box, while the other had been inoculated from a stock recently derived from the original frozen and dried material. The lesions induced by these two cultures were quite comparable in size; the average volume is recorded in Table II.

At the end of 6 weeks each of the four groups had been subjected to different sensitizing conditions. In so far as the secondary reaction is an indicator of sensitivity all groups on the 11th day seemed to be about equally hypersensitive; but the eye test showed that Group D was less so than the others. While at this early period only one animal of Group C had developed a marked, and one a slight, keratitis,

three subsequent intracutaneous injections of 10^{-4} cc. of culture at 10-day intervals apparently so increased the corneal hypersensitiveness of animals of this group that when tested on the 42nd day, three developed strongly positive ophthalmic reactions. In Group A, on the other hand, the degree of corneal sensitiveness was found to have decreased, even though the animals had received the same intracutaneous treatment as those of Group C. But by contrast the

TABLE II

Summary of Effect of Agar Foci and Repeated Intracutaneous Inoculation with 10^{-4} cc. of Culture on Maintaining the Hypersensitive State in Sensitized Rabbits

Group	No. of animals	1st day	8th day	11th day				11th day 21st " 31st "	42nd day			47th day
		Sediment of 5 cc., 5 cc., 10^{-4} cc., and 10^{-4} cc. intracutaneously	Agar focus inocu- lated with sedi- ment of 5 cc.	Secondary reaction		Ophthalmic reaction		Intracutaneous reinoculation 10^{-4} cc.	Ophthalmic reaction		Intracutaneous inoculation 10^{-4} cc. Average vol. in cmm.	Intracutaneous inoculation 10^{-4} cc. Average vol. in cmm.
				+	-	+	-		+	-		
A	27	+	+	21 78%	6	19 76%	8	Yes	12 44%	15	75	102
B	25	+	+	17 68%	8	18 72%	7	No	6 24%	19	52	64
C	6	+	0	3 50%	3	2 33%	4	Yes	3 50%	3	77	133
D	6	+	0	4 67%	2	0 0%	6	No	0 0%	6	34	62

continual sensitizing effect of these small repeated inocula is illustrated in Groups B and D, in which they had been omitted: Group B on the 42nd day gave approximately one-third the number of positive eye reactions that it did on the 11th day, and Group D failed entirely to develop sufficient hypersensitiveness for demonstration by the ophthalmic test. The comparative intensities of the skin reactions in the various groups on the 42nd and 47th days also showed that the repeated production of cutaneous foci with only 10^{-4} cc. of culture

was more favorable for the continuation of the hypersensitive state than was the production of a single subcutaneous agar focus. The stronger reactions of all of the animals on the 47th day as compared with the 42nd indicate either that the culture used for testing was more potent or that the animals were more reactive. As the two different cultures used for testing on the 47th day gave reactions of approximately the same intensity, it is probable that the cutaneous sensitivity of all of the rabbits had been elevated by the small cutaneous inoculation 5 days previously. The experiment in all of its phases indicates clearly that after animals are made hypersensitive, this state can be continued or increased by cutaneous foci induced by doses of streptococci which are incapable of stimulating macroscopically visible lesions in normal animals.

In practically all previous experiments it had been assumed that a first inoculation of the sediment of 5 cc. of culture was necessary for the development of the hypersensitive state; in other words, that a large amount of bacterial substance was the important factor in giving the initial impulse. But control groups in a number of experiments, such as Group E in Experiment 1, had shown that distinct hypersensitiveness might follow the production of three lesions with 10^{-1} cc., 10^{-2} cc. and 10^{-4} cc. of culture respectively. Moreover, in a comparison of the sensitizing capacities of a large number of strains of indifferent streptococci (3) it was found that nine small lesions often led to a marked degree of hypersensitiveness. It seemed advisable, therefore, to determine whether the total dosage of streptococci injected intracutaneously or the number of lesions produced was the more important in inducing a condition of hypersensitiveness, provided the lesions were all made at one time.

Experiment 3.—Five groups of 4 rabbits each were inoculated intracutaneously in a varying number of places with different doses of indifferent *Streptococcus* Q186A/0/6, as shown in Table III. The number of lesions varied between 1 and 11 and the total dosage was the centrifugate of either 0.222 cc., 5.0 cc., or 5.222 cc. of a 20-hour blood broth culture. All of these lesions were measured daily to detect secondary reactions; and on the 14th day the comparative hypersensitiveness of the animals was tested with intracutaneous inocula of 10^{-2} cc., 10^{-4} cc., and 10^{-6} cc. of a homologous culture, and the ophthalmic test was applied to each. A new group (F) of normal controls was introduced at this time to test the activity of the culture.

Two rabbits of Group A sickened of *B. lepirosepticum* infection; hence comparison with the others is somewhat vitiated. But the two survivors developed secondary reactions, and showed lesions on reinfection comparable with those of the other groups. Moreover, the results of the rest of the experiment do not indicate that these Group A animals would necessarily have been less sensitive than any of the others. Group E, the members of which received only a single injection of 5 cc. of centrifugate, was the most poorly sensitized. Group C, on the

TABLE III

Comparative Sensitizing Effect of Different Doses of Streptococci Distributed in a Varying Number of Lesions

Group		Amount of inoculum					Each animal		Total with secondary reaction	Test 14th day			
		5 cc.	1 cc.	10 ⁻¹ cc.	10 ⁻² cc.	10 ⁻³ cc.	Total lesions	Total dose		Positive ophthalmic reaction	Average maximum volume retest on 14th day		No. of animals showing reaction with
											10 ⁻¹ cc.	10 ⁻² cc.	
A	No. of lesions	1	...	2	2	2	7	5.222	2*	1	367**	96**	2
B	induced by	...	5	2	2	2	11	5.222	3	1	375	79	2
C	inocula of	2	2	2	6	0.222	3	2	325	55	3
D	respective	...	5	5	5.0	3	2	532	101	4
E	size	1	1	5.0	1	1	387	36	2
F													
Controls		0	85	0	0

* Only 2 rabbits of Group A survived until the 14th day.

** Figure indicates average for the group.

other hand, which received approximately only 1/20th of the dose used in the other animals but divided amongst six areas, developed hypersensitiveness in as many members as either Group B or D. The latter group, however, showed the largest lesions following reinoculation.

The culture used in this experiment was capable of producing in normal animals lesions about 1 cm. in diameter with doses of 10⁻³ cc., but no macroscopic lesions with 10⁻⁴ cc. In other experiments it has been observed that unless a particular strain could evoke fair

sized lesions with doses of at least 10^{-1} cc. or 10^{-2} cc. it was practically useless in eliciting secondary reactions or in inducing a state of hypersensitiveness of sufficient intensity to be detected by the ophthalmic test. One may conclude, therefore, that if with any suitable strain lesions of about 10 mm. diameter are produced by relatively small doses, a number of these small lesions will more readily induce a hypersensitive state in rabbits than will a single lesion made by injecting a much larger quantity.

The demonstration that intracutaneous injection of non-hemolytic streptococci at 10-day intervals, in doses 1/10th the size necessary to produce minimal macroscopic lesions in normal animals, either maintained or enhanced the hyperergic state in rabbits already somewhat hypersensitive, made it appear that possibly it was the amount of tissue injured in a peculiar manner rather than the quantity of bacterial substance which furnished the direct stimulus for hypersensitization.

The time (30 to 60 days) required by this method to render the animals sufficiently hypersensitive for further experimental procedures made it desirable to determine whether the same principle might not be applied to secure a more rapid sensitization of the animals.

Experiment 4.—The microorganism, P64, used in this experiment, was a green-forming streptococcus, which in doses of 10^{-2} cc. of blood broth culture induced in normal rabbits lesions of 10 to 15 mm. in diameter and about 1 mm. in height; these usually persisted for 1 to 3 days. Macroscopic lesions were usually not visible at the site of 10^{-3} cc. inoculation; but at the sites of injection of 1 cc. and 10^{-1} cc. there occurred long tracking zones of redness and edema which gradually disappeared, and left areas in which secondary reactions were most likely to occur. Each rabbit of two groups was inoculated intracutaneously with 1 cc., 10^{-1} cc., 10^{-2} cc., and 10^{-3} cc. of a 24-hour blood broth culture. Group A animals were simply observed in the usual manner; but those of Group B received during the next 2 weeks 11 injections each of 10^{-3} cc. of similar cultures; these inoculations were made daily with the exception of the 6th and 13th days; and each resulting lesion was measured daily until it diminished in size. On the 15th day the ophthalmic test was applied to the left eyes, and on the 22nd day all of the rabbits were inoculated intravenously with the sediment of equivalent amounts per kilo body weight of 24-hour blood broth culture. The results are summarized in Table IV.

It is obvious that daily inoculations for 2 weeks induced distinct hypersensitiveness in all of the Group B animals, while those of Group A receiving merely the original inocula failed to become sufficiently sensitive to respond to any of the three tests used to detect this state. It is not to be expected that such a clear cut difference between the two groups would always appear, for with other strains a considerable degree of hypersensitiveness has often been induced in some animals by a single series of inoculations similar to or smaller than that used in Group A. With this strain, however, we were fortunate in obtaining conditions favorable for the illustration of the advantage of the added daily inoculation of small doses.

TABLE IV
*Comparative Sensitizing Effect of Initial Inoculum and a Similar Inoculum
Followed by Daily Inoculation*

Group	Rabbit No.	Secondary reaction		Reaction from 10^{-4} cc. inoculation		15th day ophthalmic reaction	22nd day lethal reaction
		10^{-3} cc.	10^{-1} cc.	Begun	Maximum		
A	R2126	0	0	0	0
	R2127	0	0	0	0
	R2129	0	0	0	0
B	R2130	\pm 10th day	++++ 12th day	4th day	10th day	++	+
	R2131	+ 12th day	+++ 7th day	3rd day	9th day	++	+
	R2132	++++ 12th day	++++ 15th day	4th day	10th day	?	S*

* Sick, but recovered.

Another point brought out in this experiment was the early onset of hypersensitiveness, detected by measuring the 10^{-3} cc. lesions in Group B (see Chart 2). Whereas the first two or three areas inoculated with this dose showed little or no gross evidence of infection, those following the third or fourth inoculation developed distinct maculopapules, and each subsequent injection induced a lesion of greater volume than the previous one. The maximum reaction occurred in areas inoculated on the 9th or 10th days, or about the time of appearance of the secondary reactions at the sites of the original larger inoculations. Then the intensity of the reactions following

reinoculation seemed to diminish somewhat, a suggestion that an "immune state" was being superimposed on the pre-existing hyperergic condition. Similar diminution in reactivity has been observed by Julianelle (4) in sensitizing rabbits with pneumococcus vaccines.

Still another advantage is suggested for this mode of sensitization. The recording of the amount of reaction occurring at the site of each reinoculation discloses, by the end of the first week, the probable capacity of the animal to develop a satisfactory state of hypersensitive-

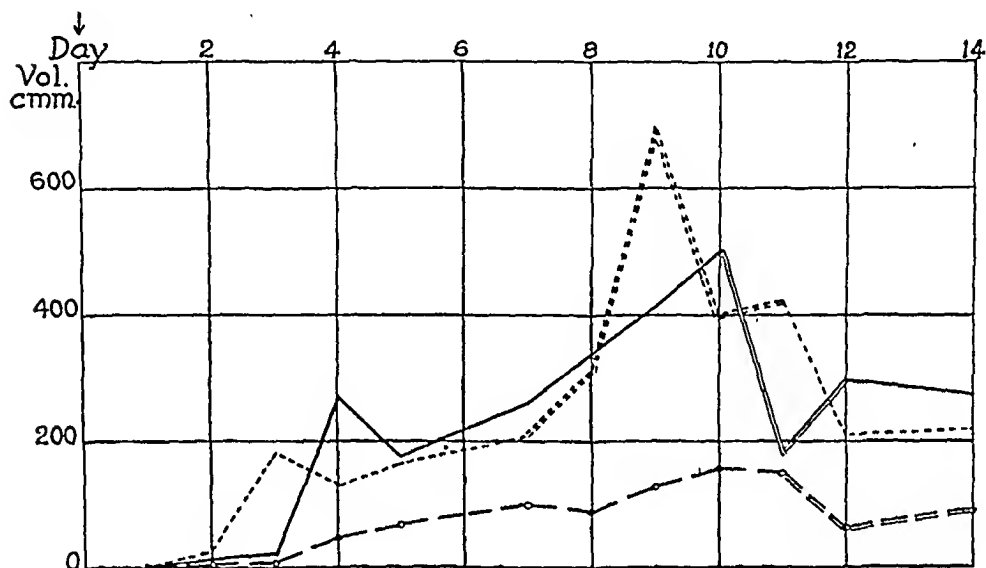


CHART 2. Volume of lesions resulting from daily inoculation with 10^{-3} cc. of *Streptococcus* P64.

Double lines indicate time of appearance of secondary reaction at site of original inoculations.

ness. This point is brought out by comparing Rabbit R2132 with the other two of the group (see Chart 2). The degree of reaction was slow in increasing, and was always small as compared with that of the other two animals; the secondary reaction was correspondingly slow in developing; the ophthalmic reaction was equivocal; and intravenous inoculation merely caused the animal to sicken without leading to death. In subsequently applying this mode of sensitization we have been able to determine within 7 to 10 days that certain animals were showing the cachectic type of response or failing to become satis-

factorily hypersensitive, and thus to eliminate them from any series to be used for comparative studies.

From Experiment 4 it was not clear whether daily inoculations with small doses of streptococci for 2 weeks without the initial larger inocula might not have been as efficacious as the two combined; hence additional experiments were performed to answer this question.

Experiment 5.—*Streptococcus* V110A was used as the sensitizing strain. The animals of Groups A and B were inoculated in a manner similar to those in Experiment 4; but those of Group C received only daily inoculations of 10^{-3} cc. amounts over a period of 2 weeks. Ophthalmic tests did not reveal sharply defined differences amongst the various groups; but in this experiment, as well as in Experiment 4, the findings at autopsy after comparable intervals following lethal tests showed the most severe and widespread lesions in animals of Group B, which had received daily small inoculations in addition to an initial series of larger doses; the members of Group C, which had been sensitized with daily inoculations alone, showed somewhat more extensive lesions than those of Group A which had received simply the initial inoculations.

Comparison of the average volumes of the reactions from repeated 10^{-3} cc. inocula showed that in the 2-week period the animals of Group B had somewhat larger lesions; hence one may conclude that in this period the strong initial stimulus combined with daily inoculation was more efficacious than the daily small inoculations alone.

Experiment 6.—In Experiment 6 the reactions to daily inoculation of 10^{-3} cc. of blood broth culture of *Streptococcus viridans* V110A were followed for 4 weeks. In Group B initial inocula of 1 cc., 10^{-1} cc., and 10^{-2} cc. were given; in Group C these were withheld, while in both groups single daily injections of 10^{-3} cc. were made. The average of the two groups is shown in Chart 3.

In all animals a wave-like rise and fall of cutaneous reactivity was observed. The curve rose most rapidly in the first 10 or 12 days; then there was a fall followed by a second peak about the 20th day, following which a second fall occurred.¹ All animals of Group B then showed a stationary or falling trend; in one this was very marked as though the animal were becoming "immune." It was impractical to carry the experiment further because most of the good available

¹ A somewhat similar wave-like variation in reactivity to repeated intracutaneous inoculation is recorded by Krause and Willis in tuberculous guinea pigs (*Am. J. Tuberculosis*, 1926, 14, 316).

skin had been utilized. This experiment demonstrates, however, that a high degree of sensitivity can be attained by repeated daily inocula of relatively small doses of non-hemolytic streptococci, when carried out over a prolonged period.

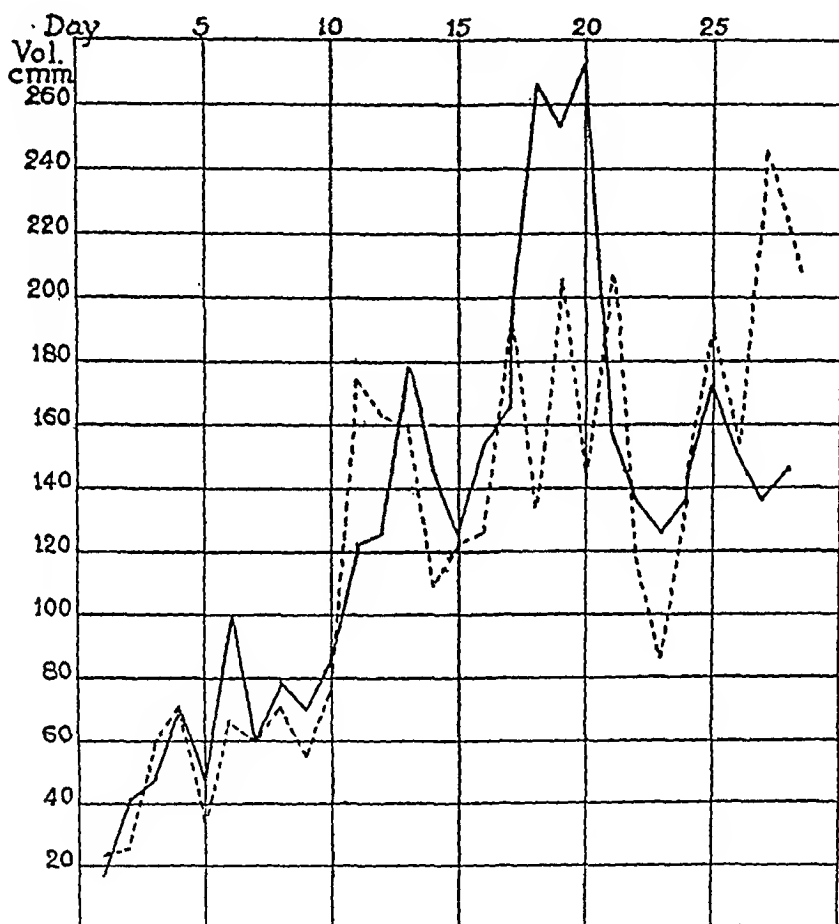


CHART 3. Comparative intensity of reactions at site of daily inoculations of 10^{-3} cc. of *Streptococcus* V110A.

Solid line, Group B received original inoculation of 1, 10^{-1} and 10^{-2} cc. followed by daily inoculation of 10^{-3} cc. Broken line, Group C received daily inoculation of 10^{-3} cc. only.

The tendency for the lesions to be less marked during certain periods in animals receiving daily inocula suggested that possibly the hypersensitive state might be temporarily depressed as a result of too

frequent inoculation; and in addition Experiment 2 suggested that after a certain degree of hypersensitivity had been attained, less frequent inoculations would serve to maintain or heighten this condition. Experiment 7, therefore, combined certain features of the two methods.

TABLE V

Comparison of Degree of Hypersensitiveness of Corneae after 2 and 9 Weeks

No. animals	Strain	After 2 weeks' sensitization. Ophthalmic reaction			After 7 weeks' additional sensitization 10 ⁻³ cc. and 10 ⁻⁴ cc. each week			
					Ophthalmic reaction			Lethal test. No. died
		—	±	+ to ++++	—	±	+ to ++++	
3	Q155	...	1	2	...	1	2	2*
3	Q167B	3	1	...	2	3
2	R1	2	1	...	1	2
4	R9	...	2	2	4	4
2	RF156	2	2	2
3	RF162B	3	2	...	1	0
3	A49	1	2	3	3
Total, 20		9	5	6	4	1	15	16
Per cent.....		45	25	30	20	5	75	80

* The other animal of the group rendered very sick, but recovered. Autopsy findings showed typical changes.

Experiment 7.—Seven different strains of streptococci, six indifferent and one green-forming, were employed; each rabbit received but a single strain, with a distribution of strains among groups as shown in Table V. The inoculation on the first day consisted of 1 cc., 10⁻¹ cc., 10⁻² cc., and 10⁻³ cc. of blood broth culture; thereafter daily inocula of 10⁻³ cc. were given for 2 weeks, at the end of which time the right corneae were inoculated with homologous streptococci. For the next 7 weeks each animal received 10⁻³ cc., and 10⁻⁴ cc. of homologous culture every 7 days. The left eyes were then tested; and 1 week later all animals were inoculated intravenously with the usual lethal test dose of the homologous strain. The results are summarized in Table V.

It is evident that the 7 weeks' additional treatment greatly increased the hypersensitiveness of the entire group. While somewhat more than one-half of the animals showed some degree of corneal sensitivity

at the end of 2 weeks, in one-fourth it was only slight. Seven weeks later, however, the number of strong ophthalmic reactions had more than doubled. The lethal test, moreover, indicated that all of the animals except those inoculated with Strain RF162B had been rendered very hypersensitive by the prolonged treatment; this was a much larger number of positive lethal reactions than would have been expected at the end of 2 weeks in a group of animals showing so few strongly positive ophthalmic reactions. In fact, if the animals inoculated with Strain RF162B be deleted from the experiment, and the one severely but not fatally shocked animal of the Q155 group be included, it can be said that all of the rabbits inoculated with six of the strains showed at the end of 9 weeks marked general hypersensitivity. The RF162B group illustrates the necessity of employing suitable strains in order to obtain satisfactory sensitivity, for although these animals were treated in identically the same manner as the others, their skin reactions were always poor and none of them was rendered sufficiently sensitive to sicken after intravenous inoculation.

DISCUSSION

Several interesting points have emerged during the progress of this work. At the beginning it was thought that the degree of hypersensitiveness would be more or less proportional to the size of the initial dose. This was to be expected from the work of Roemer (5) Krause (6) and others on hypersensitiveness in experimental tuberculosis, and of Bloch (7) and his co-workers in trichophyton infections. But it soon became evident that, within certain limits, the initial production of multiple lesions with smaller doses of non-hemolytic streptococci was more likely to lead to marked hypersensitivity than that which followed a single lesion induced by larger amounts; furthermore, the most satisfactory hypersensitiveness followed repeated inoculations with small doses of the microorganisms. In a number of instances these doses were too small to induce macroscopic lesions in normal animals.

The results of infection of animals with single inocula of non-hemolytic streptococci and with tubercle bacilli are not exactly comparable. In the first instance, as the microorganisms are so quickly killed they do not multiply long at the site of inoculation and are dis-

seminated to other areas for only a short time. In the second case they grow and are carried to distant organs, where they again increase and produce lesions; hence in the first instance relatively few local lesions are produced, while in the second case multiple and disseminated injuries occur. Repeated small inoculations with streptococci, therefore, probably reproduce more nearly the conditions following a single inoculation with tubercle bacilli. This view point is substantiated by the results of implanting agar foci infected with streptococci. Such foci harbor living streptococci for a period of from 3 to 4 weeks, but even after becoming sterile they presumably contain disintegrated bacterial sensitizing products for long periods. Nevertheless these foci have not proven as efficacious in increasing the sensitive state as have repeated inocula of small size. This suggests that the kind of lesion rather than the amount of bacterial substance is the important factor. Indeed, lesion—tubercle production—is the *sine qua non* for the development of tuberculin allergy; and it is highly suggestive that Zinsser and Mueller (8) found that the production of multiple skin tuberculous lesions in rabbits yielded the best conditions for passive transfer of tuberculin allergy. They stress the rôle of the focus in the induction of "allergy;" but later Zinsser (9) thought that the peculiar manner in which the bacteria are disintegrated was the important factor. Whatever may be formed from the bacteria in a focus, our experiments indicate that only exceedingly small amounts of the substance are necessary to increase the hypersensitive condition, once the initial stimulus has been provided for the development of that state.

Experience with various infections indicates that there are two types of reaction towards reinfection with microorganisms—that of hypersensitivity, and that of immunity. That the two are often intimately linked is indicated by the opinions of most workers in this field. But there is a growing tendency to regard the two processes as not necessarily interdependent (Boquet and Nègre (10), Swift and Derick (1, 2), Rich (11)). One problem of experimental investigation is to determine the best manner of imposing these two types of reaction, each independently of the other, on different animals, in order to study their respective rôles in the general subject of resistance. Julianelle (4) noted that rabbits sensitized by intracutaneous injection of vaccines of pneumococci showed increasing lesions up to a certain time;

there was then a decrease which was associated with the appearance of immune bodies in the serum. Panton and Valentine (12) observed that rabbits receiving repeated inoculations with living staphylococci eventually showed decreasing reactions to large inocula and increasing reactions to small ones, another example of concomitant hypersensitiveness and immunity. Both groups of observers employed relatively large inocula; although the latter stated that hypersensitiveness could be induced by small doses.

From a theoretical standpoint it might be expected that when relatively large amounts of bacterial substances are acting within an animal's body there is more possibility of wide distribution and accompanying immunity. With small amounts, there is simple focal lesion-production, and attending hypersensitivity. Our experiments indicate that repeated small focal lesions usually lead to increasing general tissue hypersensitivity without local evidence of immune tissue response. Unfortunately the relative avirulence of the non-hemolytic streptococci makes it impossible to test the general immunity of rabbits so sensitized; but we have observed that rabbits may be highly sensitized and show little or no serum agglutinin against the sensitizing microorganism, and little precipitin against its nucleoprotein. Contrasted with this may be cited the observations of Fleischer and Meyer (13) that following intraperitoneal injections of vaccines prepared from *B. abortus* or *B. typhosus*, guinea pigs would yield high agglutinins against the homologous bacterium without giving abortin or typhoidin reactions.

The influence of chronicity of infection as a sensitizing factor is indicated by a number of our experiments; and again let it be emphasized that the dose of bacteria sufficient to increase the hypersensitive state was too small to induce macroscopic lesions in normal animals. Thus in the hypersensitive animal there are set up conditions favorable for the augmentation of this state in the presence of a minimum dose of antigen. Klinge (14) has recently emphasized the importance of chronicity in the production of certain peculiar reactions to coagulable protein; while the observations of Dienes (15) suggest that the local condition of the tissue into which a coagulable protein is introduced may also determine the subsequent type of reaction to that protein. Both factors doubtless have important influences, but in order to

evaluate them the importance of dosage of antigen must be considered more carefully than in the past. In experimentally inciting the hypersensitive state (tuberculin-like allergy or hypersensitiveness of infection) large doses of bacteria have usually been employed; but in natural infections characterized by so-called "allergy"—better "hyperergy"—the infecting dose of microorganisms is usually small, and presumably the inciting of new lesions at a distance from the initial lesions is usually effected by relatively few germs which doubtless multiply to a certain extent at the site of metastatic implantation. But in syphilis, which is a classical example of changing "allergic" reactions, it is noteworthy that the most destructive lesion, the gumma, usually arises only after the spirochetes have been acting in the body for a number of years; and at times antecedent evidences of infection—in other words, lesions—may have been so small as to escape detection. Other clinical examples of "allergic alteration" of tissues might be cited to show that in nature the hypersensitive state is induced or maintained by small lesions existing or reproduced over considerable periods of time.

SUMMARY

The most satisfactory method thus far found for the induction and maintenance of a high degree of hypersensitiveness—"allergy," "hyperergy"—against non-hemolytic streptococci consists in the repeated production of small focal lesions with minimal doses of bacteria.

After a preliminary sensitizing period of about 2 weeks' duration with either large initial, or small multiple daily inoculations, the later foci need be produced only at 7 to 10 day intervals.

Chronicity of low-grade infection appears to be an important factor in the attainment of a high degree of hypersensitiveness.

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STUDIES IN THE BLOOD CYTOLOGY OF THE RABBIT

II. CONSECUTIVE ERYTHROCYTE AND HEMOGLOBIN OBSERVATIONS ON GROUPS OF NORMAL RABBITS

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In the first paper of this series dealing with the blood cytology of the rabbit, the results of 1110 blood counts on 174 normal male rabbits were considered primarily from the standpoint of the numerical variations of the various classes of cells and of the hemoglobin content in individual determinations (1). Other phases of this investigation have included repeated observations on groups of normal and diseased rabbits for extended periods of time. In the case of normal animals, the study was undertaken chiefly for two reasons. Firstly, information was desired concerning the character of the spontaneous fluctuations in the numbers of blood cells under prolonged conditions of undisturbed indoor life with the idea that eventually some relationship might be found between these variations and certain environmental conditions, as was demonstrated with the weights of organs and the amount and degree of change of the total sunlight (2). Secondly, it was considered essential to have information of this nature as a background for whatever changes might be found in association with certain subacute or chronic experimental conditions, as for example, *Treponema pallidum* infection. Five groups of rabbits, 4 of 10 and 1 of 5 animals, were followed 35, 13, 8, 29, and 26 weeks respectively, covering a period of 20 months from October, 1927 to July, 1929. The group examinations numbered 111, in which 973 individual counts are represented.

The results of this study which have been analyzed statistically, will be reported in the present and subsequent papers. For the time being, the principal interest centers in the consecutive mean values of the various classes of cells considered from the standpoint of the trends

of numerical levels shown by each animal group. In the present paper, the erythrocytes and the hemoglobin content are considered; other papers will deal with the total white cells and their division into granular and non-granular cells (3), with the neutrophiles (pseudo-eosinophiles), the basophiles, and the eosinophiles (4), and with the lymphocytes and the monocytes (5). The results will ultimately be analyzed from the standpoint of the relationships of the various classes of cells as shown by their correlation coefficients and in addition, they will be referred to in connection with the relation of the pre-inoculation blood picture to the reaction of the host to disease agents.

Materials and Methods

The rabbits employed were representative of those used in other experiments carried out in this laboratory; all were male animals approximately 6 to 8 months old. The ordinary brown and grey type predominated but the type described as the Flemish cross or mixture was also represented and there were a few black animals. Each rabbit was separately caged in a well lighted (sunlight), well ventilated room; the diet throughout the period of observation consisted of hay, oats, and cabbage.

The results reported are based upon 111 group examinations carried out at weekly intervals; in a few instances, the counts were made at shorter or longer intervals. The total number of individual blood counts was 973. The observations were made on 45 rabbits comprising 4 groups of 10 and 1 group of 5 animals:

Group	Number of rabbits	Number of examinations	Number of counts	First count	Last count
I	10	35	350	Oct. 24, 1927	June 20, 1928
II	10	13	130	Mar. 29, 1928	June 19, 1928
III	10	8	80	Sept. 20, 1928	Nov. 22, 1928
IV	10	29	283	Nov. 27, 1928	June 18, 1929
V	5	26	130	Dec. 29, 1928	June 21, 1929
	45	111	973		

A uniform routine with respect to the time and the method of collection and examination of the blood was followed as previously described (1). In the case of Group I, half of the animals were examined on one day and half on the following day of each week; all animals in each of the other groups were examined on the same day. The differential white blood determinations were made with the supravital neutral red technic; 100 cells were counted in each specimen.

In the case of Group I, additional specimens (10 cc.) of blood were taken for

chemical examination. During the first 2 months of the experiment, this procedure was carried out at weekly intervals; from January to April, the period was extended to 2 weeks, and the last bleeding on May 17 was made 4 weeks after the preceding one. These bleedings always followed the blood counts by 1 or 2 days in order that as long a time as possible would elapse before the next blood count.

In the analysis of results, absolute numbers of cells per cubic millimeter of blood have been used. The curves obtained by plotting the actual group means were smoothed by the formula $\frac{a + 2b + c}{4}$, the initial value of each curve being represented by $\frac{2b + c}{3}$ and the final value by $\frac{a + 2b}{3}$. The coefficients of variation of the means have also been smoothed by this formula. The smoothed mean values have been compared, in the form of percentage deviations, with the mean numbers of cells found in 1110 normal blood counts (1). For convenience, certain figures used in this comparison as so-called standard values differ slightly from the actual results obtained, as shown in the following tabulation:

	Actual values	Values used
	<i>per cmm.</i>	<i>per cmm.</i>
Red blood cells.....	5,198,000	5,200,000
Hemoglobin.....	63%	63%
White blood cells.....	9562	9560
Neutrophiles.....	4341	4340
Basophiles.....	950	950
Eosinophiles.....	214	215
Lymphocytes.....	3045	3050
Monocytes.....	1000	1000
Total granular cells.....	5504	5505
Total non-granular cells.....	4045	4050

It should be pointed out that the results of the first 3 groups of animals here reported representing 560 counts, appear among the 1110 counts of the above tabulation; the 413 counts of the 4th and 5th groups, on the other hand, are not included in the large group.

The figures as given include all data. No count has been omitted because of the occurrence of such conditions as snuffles or ear canker in certain animals at some time during the period of observation, but these instances will be referred to in the discussion of the results.

RESULTS

The results pertaining to the consecutive weekly erythrocyte counts and the hemoglobin contents obtained in 5 groups of normal rabbits

TABLE I

Group I—10 Rabbits. *Consecutive Values for Erythrocytes and Hemoglobin*

Date	Red blood cells (000 omitted)			Hemoglobin		
	Mean values	Standard deviation	Coefficient of variation	Mean values	Standard deviation	Coefficient of variation
1927-28	per cmm.	per cmm.	per cent	per cent	per cent	per cent
Oct. 24*.....	5420 ± 192	901	16.62	54 ± 2.1	10	18.52
Nov. 1.....	5539 ± 42	199	3.59	49† ± 0.4	2	4.08
Nov. 8**.....	4991 ± 79	372	7.45	55 ± 1.4	7	12.73
Nov. 15.....	5039 ± 92	433	8.59	59 ± 1.3	6	10.17
Nov. 22.....	5204 ± 130	611	11.74	52 ± 1.3	6	11.54
Nov. 29.....	5351 ± 76	355	6.63	63 ± 1.3	6	9.52
Dec. 6.....	5323 ± 229	576	10.82	63 ± 2.1	10	15.87
Dec. 13.....	5075 ± 143	670	13.20	60 ± 1.4	7	11.67
Dec. 20.....	5032 ± 170	799	15.88	60 ± 1.4	7	11.67
Dec. 27.....	5296 ± 118	555	10.48	59 ± 1.4	7	11.86
Jan. 3.....	5286 ± 129	605	11.45	57 ± 2.3	11	19.30
Jan. 10.....	4889 ± 115	540	11.05	64 ± 1.4	7	10.94
Jan. 17.....	4859 ± 182	851	11.81	61 ± 1.4	7	11.48
Jan. 24.....	5513 ± 126	591	10.72	67 ± 1.4	7	10.45
Jan. 31.....	5399 ± 176	825	15.28	64 ± 1.7	8	12.50
Feb. 7.....	5474 ± 145	679	12.40	68 ± 1.3	6	8.82
Feb. 14.....	5204 ± 153	719	13.82	68 ± 1.3	6	8.82
Feb. 21.....	5336 ± 156	730	13.68	71 ± 1.3	6	8.45
Feb. 28.....	5312 ± 178	834	15.70	74 ± 1.0	5	6.76
Mar. 6.....	5158 ± 101	475	9.21	69 ± 1.7	7	10.14
Mar. 13.....	5451 ± 155	725	13.30	63 ± 1.3	6	9.52
Mar. 20.....	5024 ± 116	544	10.83	67 ± 1.3	6	8.96
Mar. 27.....	5229 ± 200	936	17.90	72 ± 2.1	10	13.89
Apr. 3.....	4838 ± 134	628	12.98	66 ± 1.9	9	13.64
Apr. 10.....	5273 ± 96	451	8.55	68 ± 2.1	10	14.71
Apr. 17.....	5455 ± 83	388	7.11	64 ± 1.0	5	7.81
Apr. 24.....	5050 ± 180	844	16.71	58 ± 1.9	9	15.52
May 1.....	5310 ± 148	696	13.11	64 ± 1.4	7	10.94
May 8.....	5560 ± 117	549	9.87	66 ± 2.3	11	16.67
May 15.....	5188 ± 110	515	9.93	66 ± 1.7	8	12.12
May 22.....	5335 ± 205	962	18.03	66 ± 2.1	10	15.15
May 29.....	5597 ± 162	761	13.60	64 ± 1.0	8	12.50
June 5.....	6080 ± 205	959	15.77	66 ± 1.3	6	9.09
June 12.....	5730 ± 110	518	9.04	68 ± 1.9	9	13.24
June 19.....	5779 ± 111	522	9.03	69 ± 0.8	4	5.80
Mean.....	5303 ± 30	263	4.96	64 ± 0.7	6	8.59
Minimum.....	4838			52		
Maximum.....	6080			74		

* October 24 and 26.

** November 4 and 9.

† Mean of 5 specimens.

TABLE II

Group II—10 Rabbits. Consecutive Values for Erythrocytes and Hemoglobin

Date	Red blood cells (000 omitted)			Hemoglobin		
	Mean values	Standard deviation	Coefficient of variation	Mean values	Standard deviation	Coefficient of variation
1925	per cmm.	per cmm.	per cent	per cent	per cent	per cent
Mar. 29.....	4912 \pm 87	407	8.29	66 \pm 1.3	6	9.09
Apr. 3.....	4899 \pm 69	323	6.59	65 \pm 1.4	4	6.15
Apr. 10.....	4842 \pm 62	289	8.84	66 \pm 1.0	5	4.55
Apr. 17.....	5105 \pm 163	762	14.93	64 \pm 1.7	7	10.94
Apr. 24.....	5197 \pm 83	387	7.45	60 \pm 1.7	7	11.67
May 1.....	5169 \pm 139	653	12.63	64 \pm 1.7	7	10.94
May 8.....	5348 \pm 119	558	10.43	63 \pm 1.3	6	9.52
May 15.....	5047 \pm 85	400	7.93	63 \pm 1.7	6	9.52
May 22.....	5242 \pm 96	450	8.58	66 \pm 1.7	7	10.61
May 29.....	5270 \pm 107	500	9.49	63 \pm 1.7	7	11.11
June 5.....	5648 \pm 116	523	9.26	69 \pm 1.3	6	8.70
June 12.....	5329 \pm 123	575	10.79	61 \pm 1.3	6	9.84
June 19.....	5151 \pm 90	421	8.17	64 \pm 1.7	8	12.50
Mean.....	5166 \pm 39	209	4.05	64 \pm 0.4	2	3.13
Minimum.....	4899			60		
Maximum.....	5648			69		

TABLE III

Group III—10 Rabbits. Consecutive Values for Erythrocytes and Hemoglobin

Date	Red blood cells (000 omitted)			Hemoglobin		
	Mean values	Standard deviation	Coefficient of variation	Mean values	Standard deviation	Coefficient of variation
1928	per cmm.	per cmm.	per cent	per cent	per cent	per cent
Sept. 20.....	5039 \pm 34	161	3.20	70 \pm 1.7	8	11.43
Sept. 28.....	5129 \pm 51	239	4.66	56 \pm 1.0	5	8.93
Oct. 10.....	5075 \pm 23	106	2.09	70 \pm 1.0	5	7.14
Oct. 19.....	5116 \pm 23	107	2.09	71 \pm 1.7	8	11.27
Nov. 2.....	5108 \pm 51	240	4.70	56 \pm 1.0	5	8.93
Nov. 9.....	5108 \pm 32	151	2.96	66 \pm 1.4	7	10.61
Nov. 16.....	5091 \pm 41	191	3.75	65 \pm 1.0	7	10.77
Nov. 22.....	5252 \pm 33	153	2.91	68 \pm 1.3	6	8.82
Mean.....	5115 \pm 15	62	1.21	65 \pm 1.4	6	9.23
Minimum.....	5039			56		
Maximum.....	5252			71		

TABLE IV

Group IV—10 Rabbits. Consecutive Values for Erythrocytes and Hemoglobin

Date	Red blood cells (000 omitted)			Hemoglobin		
	Mean values	Standard deviation	Coefficient of variation	Mean values	Standard deviation	Coefficient of variation
<i>1928-29</i>	<i>per cmm.</i>	<i>per cmm.</i>	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>
Nov. 27.....	5694 ± 141	660	11.59	68 ± 2.1	10	14.83
Dec. 4.....	5274 ± 107	502	9.52	64 ± 1.4	7	10.28
Dec. 11.....	5549 ± 127	595	10.72	67 ± 1.9	9	13.33
Dec. 18.....	5375 ± 53	247	4.60	61 ± 1.2	6	9.50
Dec. 26.....	5285 ± 83	389	7.35	69 ± 1.7	8	11.31
Jan. 2.....	5274 ± 107	504	9.55	64 ± 2.0	9	14.68
Jan. 8.....	4839 ± 65	306	6.32	55 ± 1.2	6	10.18
Jan. 15.....	4860 ± 62	291	5.98	59 ± 1.3	6	10.61
Jan. 22.....	4810 ± 44	206	4.27	59 ± 1.9	9	15.11
Jan. 29.....	4941 ± 68	317	6.42	54 ± 1.0	5	8.25
Feb. 5.....	4705 ± 69	322	6.84	58 ± 1.2	6	9.81
Feb. 13.....	5028 ± 57	266	5.29	60 ± 1.1	5	8.60
Feb. 19.....	4914 ± 86	405	8.24	61 ± 1.3	6	10.18
Feb. 26.....	5034 ± 53	247	4.90	60 ± 1.2	6	9.16
Mar. 12.....	4897 ± 52	244	4.98	63 ± 1.6	8	11.85
Mar. 19.....	4943 ± 66	311	6.28	60 ± 0.9	4	7.05
Mar. 26.....	4875 ± 61	284	5.83	59 ± 0.8	4	6.75
Apr. 2.....	4820 ± 35	165	3.42	60 ± 1.2	5	9.00
Apr. 9.....	4774 ± 55	256	5.35	58 ± 1.1	5	8.68
Apr. 16.....	4849 ± 62	292	6.02	62 ± 1.4	7	10.50
Apr. 23.....	4874 ± 49	229	4.69	61 ± 1.1	5	8.75
Apr. 30.....	4806 ± 33	155	3.22	64 ± 1.1	5	7.94
May 7.....	4907 ± 40	189	3.86	64 ± 0.7	3	5.06
May 14.....	4816 ± 28	123	2.55	62 ± 1.6	7	11.34
May 21.....	4808 ± 40	177	3.68	64 ± 1.4	6	9.91
May 28.....	4843 ± 65	287	5.92	59 ± 1.7	8	13.05
June 4.....	4843 ± 56	249	5.14	66 ± 1.8	8	11.82
June 11.....	4936 ± 38	171	3.46	63 ± 2.4	11	17.00
June 18.....	4888 ± 47	197	4.03	56 ± 1.4	6	10.16
Mean.....	4981 ± 31	245	4.93	61 ± 0.5	4	5.75
Minimum.....	4705			54		
Maximum.....	5694			69		

observed 8 to 35 weeks are presented in Tables I to V in the form of group means, together with the probable errors of the means, the standard deviations, and the coefficients of variation. The series of

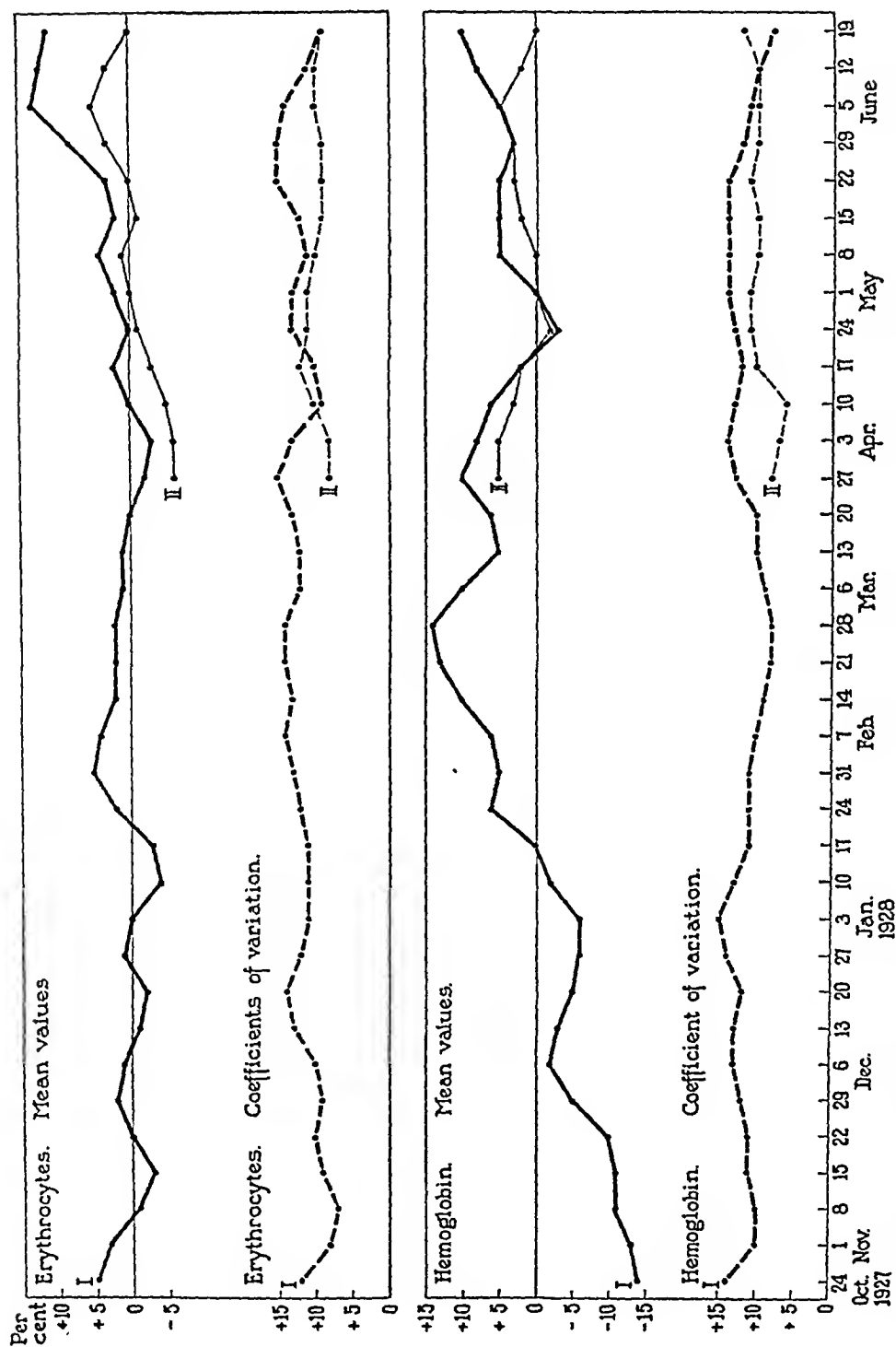
TABLE V

Group V—5 Rabbits. Consecutive Values for Erythrocytes and Hemoglobin

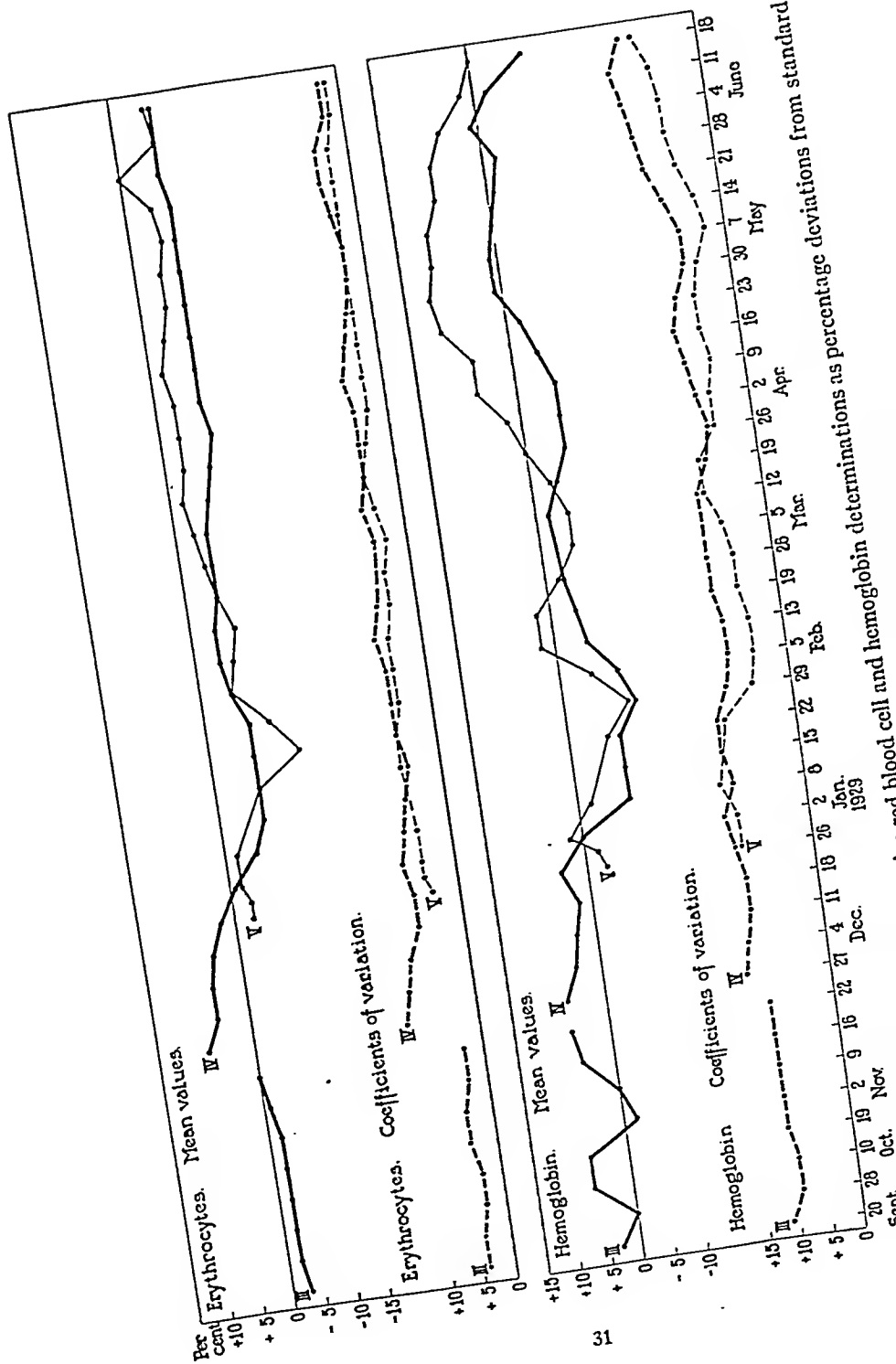
Date	Red blood cells (000 omitted)			Hemoglobin		
	Mean values	Standard deviation	Coefficient of variation	Mean values	Standard deviation	Coefficient of variation
<i>1928-29</i>	<i>per mm.</i>	<i>per mm.</i>	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>
Dec. 29	5100 \pm 57	190	2.73	60 \pm 1.8	6	10.00
Dec. 31	4920 \pm 90	297	6.04	62 \pm 2.4	8	12.90
Jan. 3.....	5224 \pm 84	279	5.34	64 \pm 1.5	5	7.81
Jan. 10.	5108 \pm 65	214	4.19	64 \pm 3.3	11	17.19
Jan. 24.....	4904 \pm 82	271	5.53	57 \pm 1.8	6	10.53
Jan. 31.....	4384 \pm 97	323	7.37	56 \pm 0.6	2	3.57
Feb 7.....	4592 \pm 75	249	5.42	58 \pm 0.9	3	5.17
Feb. 15.....	5216 \pm 54	178	3.41	67 \pm 0.9	3	4.48
Feb. 21.....	4730 \pm 106	352	7.44	64 \pm 1.2	4	6.25
Mar. 1.....	4850 \pm 35	117	2.41	61 \pm 0.6	3	4.92
Mar. 8.....	4894 \pm 78	258	5.27	61 \pm 1.2	4	6.56
Mar. 15.....	5072 \pm 37	124	2.44	58 \pm 1.8	6	10.34
Mar. 22.....	4990 \pm 47	156	3.13	61 \pm 1.8	6	9.84
Mar. 29.....	5228 \pm 86	286	5.47	64 \pm 0.9	3	4.69
Apr. 5.....	4940 \pm 58	192	3.89	62 \pm 1.2	4	6.45
Apr. 12.....	5078 \pm 50	164	3.23	69 \pm 0.9	3	4.35
Apr. 19.....	5030 \pm 33	108	2.15	66 \pm 1.2	4	6.06
Apr. 26.....	5078 \pm 57	189	3.72	70 \pm 1.5	5	7.14
May 3.....	5128 \pm 32	105	2.05	71 \pm 0.9	3	4.23
May 10.....	4882 \pm 60	198	4.06	68 \pm 0.6	2	2.94
May 17.....	4984 \pm 39	130	2.61	70 \pm 0.6	2	2.86
May 24.....	4966 \pm 49	161	3.24	67 \pm 1.5	5	7.46
May 31.....	4952 \pm 42	138	2.79	67 \pm 1.5	5	7.46
June 7.....	5050 \pm 42	140	2.77	70 \pm 1.5	5	7.14
June 14.....	4756 \pm 29	96	2.02	60 \pm 1.2	4	6.67
June 21.....	5010 \pm 33	108	2.16	64 \pm 2.1	7	10.94
Mean.....	4964 \pm 25	188	3.79	64 \pm 0.5	4	6.25
Minimum.....	4384			56		
Maximum.....	5228			71		

curves in Text-figs. 1 and 2 are drawn as percentage deviations of the smoothed means from the standard values given above; other curves represent the smoothed values of the coefficients of variation of the means.

By using mean values to describe the results obtained, the trends or



TEXT-FIG. 1. Mean values for consecutive red blood cell and hemoglobin determinations as percentage deviations from standard values. 1927-28.



changes characteristic of the group as a whole are shown. It is this feature of the results rather than the variations of individual animals which at present we wish to emphasize.

DISCUSSION AND SUMMARY

In discussing the results of repeated observations on the erythrocytes and hemoglobin content of the peripheral blood of normal rabbits over long periods of time, it will be convenient to consider in chronological order the findings obtained in each of the five groups.

Group I, comprising 10 rabbits, was examined weekly from October 24, 1927 to June 21, 1928 (Table I). The mean numbers of red cells in 35 examinations were found to be comparatively constant.

The fluctuations in the curve of the percentage deviations of the smoothed means from a standard value of 5,200,000 cells per cubic millimeter (Text-fig. 1) do not vary more than 5 per cent above or below this figure until May and June when the curve rises to the 10 to 13 per cent level. In general, the curve describes 3 major swings, each occupying a period of approximately 3 months. From the October level of 5 per cent above the base line, there is an irregular fall to 4 per cent below it on January 10. During the succeeding 3 weeks, the curve rises abruptly to its initial level and very gradually descends to slightly below the base line (March 27 and April 3). During the last 3 months the curve rises to the highest levels observed although at its termination, its trend is again downward. The coefficients of variation for the red cells means (Table I) are fairly uniform, the curve of the smoothed values varying about the 10 per cent level (Text-fig. 1). The most irregular portion of the curve is its last quarter and this phase accompanied an augmentation of mean values. It will be noted that the changes in the trend of the red cell means at the end of January and the first of April are, in both instances, preceded by similar changes in the coefficient curve, at a 2 or 3 weeks' interval.

The range of mean hemoglobin values of Group I (Table I) was considerably greater than that of the erythrocyte means. The curve of these values in terms of the percentage variations of the smoothed means from a standard of 63 per cent fluctuates within a total range of 28 per cent, that is, 14 per cent above and below the base line (Text-fig. 1).

This curve can be divided into 3 major swings which, however, are not parallel with those of the red cells. From an initial low level the curve rises to 14 per cent above the base line by the end of February; the trend of the red cell curve in the

opposite direction ended the middle of January and for the next 3 observations, the direction of both curves is upward. From the end of February to the last of April, the hemoglobin curve is generally falling, while in May and June its general movement is again in an upward direction. In March, both the hemoglobin and red cell curves are falling and from the middle of April to the end of June both are rising. The coefficients of variation of the hemoglobin means (Table I) are comparable as far as order of magnitude is concerned, to those of the red cell means. From the curve representing the smoothed coefficients (Text-fig. 1) it will be noted that small values prevailed during January and February in which months the means were considerably increased. In like manner, the second period of higher hemoglobin mean values in May and June was characterized by a steady decline of the coefficient curve.

The second group of 10 rabbits (Group II) was examined during the last 3 months of the observation period of Group I (Table II). The values for the mean numbers of erythrocytes and for the mean hemoglobin contents occupy slightly lower levels than those for Group I (Text-fig. 1), but it will be noted that the curves representing these values are remarkably like those of Group I in general outline, the only important exception being in the case of the hemoglobin means during the last 2 weeks' observations.

It should be noted that in the corresponding portion of the curve representing the coefficients of variation of these means, an abrupt upward turn occurs indicating that the sharp fall in the mean values is due to certain individual findings and is not characteristic of the group as a whole. The coefficients of variations of both erythrocyte and hemoglobin means of Group II (Table II) are generally somewhat smaller than those of Group I as is illustrated by the curves shown in Text-fig. 1.

The third group of 10 rabbits (Group III) was examined in September, October, and November of 1928. The erythrocytes and the hemoglobin means, together with their coefficients of variations are given in Table III and the curves representing these values appear in Text-fig. 2.

In the case of the red blood cells, the means were extremely regular at a level slightly below the standard value while the curve of their smoothed coefficients of variation is steadily maintained at the 3 to 4 per cent level. The hemoglobin means, on the other hand, were less uniform and their curve describes several fluctuations which, however, are of a much smaller magnitude than those of Group I. In contrast to these variations, however, the curve of the smoothed coefficients of variation of these means is almost a straight line at the 10 per cent level, a value slightly smaller than that which generally obtained in Group I.

Examination of the fourth group of 10 rabbits was begun in November, 1928 and of the fifth group comprising 5 rabbits in December, 1928; both groups were observed to the end of June, 1929. The erythrocyte and hemoglobin means and their coefficients of variations are given in Tables IV and V, and the curves illustrating these values in Text-fig. 2.

In the case of Group IV, the red cell curve which shows practically no irregularities, describes during the first 2 months' observation a steady downward trend from an initial 7 per cent above to 7 per cent below the standard value; during the remainder of the experiment this low level is almost perfectly maintained. The curve of the smoothed coefficients of variation of the red cell means has a similar form; from an initial 11 per cent level, it falls to 5 per cent at the end of the second month and to 3 per cent in June.

The hemoglobin means of Group IV were much less uniform than the red cell values as was the case with the previous groups; the fluctuations of the curve representing these values (Text-fig. 2) vary between 6 per cent above to 11 per cent below the base line. During the first 2 months, the direction of the curve is downward, parallel to that of the red cells, but from this time onward, while the red cell means continue at a fairly constant level, the general trend of the hemoglobin means is upward. The rise is interrupted in March and again in June; on the first occasion there is also a fall in the number of red cells but on the second, the red cell level is unchanged. The smoothed coefficients of variation of the hemoglobin means for Group IV vary from 7 to 14 per cent (Text-fig. 2). Their curve is fairly stationary at the 11 to 12 per cent level during the first 2 months' observations; in subsequent months somewhat lower and more irregular values obtain, and in May and June a rise to 12 and 14 per cent is observed.

The curve representing the erythrocyte means of Group V differs but little from that of Group IV as shown in Text-fig. 2. It begins in December at approximately the same level which Group IV occupies at this time and ends at the same level in June. There is an initial abrupt fall to 14 per cent below the base line (end of January) which is followed by a sharp rise, and from then onward, a fairly constant level is maintained at 5 per cent below the standard value. During January and February, the general level of the curve is slightly lower than that of Group IV while in March, April, May, and June, the relation is reversed. On the whole, the curve is uniform and contains but two irregular portions, one the end of January when the downward trend is prolonged to the lowest level observed in any of the 5 groups, that is, 13 per cent below the standard value, and the other the first of June at which time there is a sharp but temporary upward movement. The curve representing the smoothed coefficients of variation of the red cell means which is extremely uniform (Text-fig. 2) occupies a general level slightly below that of Group IV. It describes a gradual fall from 6 to 2 per cent.

In the case of the hemoglobin content, the mean values of Group V were somewhat higher than those of Group IV, but the direction of the variations observed

was essentially the same (Text-fig. 2). The curves representing these values are similar in general contour with parallel fluctuations but those of Group IV are somewhat more pronounced, their extremes being 10 per cent below and 11 per cent above the standard value. The level of the curve representing the smoothed coefficients of variation of the hemoglobin means of Group V is generally lower than that of Group IV, as was the case with the red cells (Text-fig. 2); the fluctuations in magnitude vary from 3 to 13 per cent. The general trend of this curve is similar to that of Group IV.

The great majority of the rabbits in these experiments were in excellent physical condition and gained in weight during the period of observation. There were two deaths, both in Group IV, which occurred toward the end of the experiment. Both animals had had marked snuffles and at autopsy, an extensive purulent exudate was found in the nasal passages and sinuses and in one rabbit, there was also a chronic diffuse interstitial nephritis. Snuffles and ear canker of various grades which were observed in certain rabbits will be referred to in a subsequent paper in connection with the discussion of the observations on the neutrophile cells (4).

There are certain features of these consecutive observations on the peripheral blood of normal rabbits which may now be briefly considered. In the first place, the weekly range of variation of the erythrocyte mean counts was considerably smaller than that of the hemoglobin means, a result which indicates that under the conditions of these experiments, the hemoglobin was the more labile of these two blood constituents. The technic employed may have contributed to this finding in that the error involved in a colorimetric method may be greater than in one of cell enumeration. With each specimen of blood, at least 3 hemoglobin readings were made and the mean value used; and in addition, duplicate readings with another instrument have always checked with those of our own. Secondly, it was found that a brief or a more sustained alteration in the erythrocyte means was not necessarily accompanied by a shift of the hemoglobin level in the opposite direction. In certain instances, such a change was observed, but in others, both values moved in the same direction and in still others, the hemoglobin means pursued a more or less protracted rise while the numbers of red cells were maintained at a fairly stationary level. Thirdly, the most irregular results with respect to both red cell and hemoglobin means were found in Group I and in addition,

their standard deviations were on the whole, of a higher order of magnitude than those of the other groups (Tables I to V). The examinations on Group I were not all made on the same day, half the animals being examined on one day and half on the following day; in the case of the other groups, all animals were examined on the same day. As has been mentioned in the section on Materials and Methods, the rabbits of Group I were also subjected to additional bleedings for purposes of chemical examination. Although this procedure was carried out most frequently in the first 2 months of the experiment, there is no striking difference in the order of magnitude of the standard deviations of the means nor in the coefficients of variations during this period as compared with later months (Table I). It is probable, therefore, that the greater irregularities of mean values and the higher standard deviations noted in Group I were related to the divided time of the blood counts themselves or to some other factor rather than to the additional bleedings for chemical examination.

In the present consideration, the major changes in mean values as observed over several weeks or months have been emphasized rather than fluctuations occurring in comparatively brief periods. Analysis of the results from the standpoint of the ratio of the difference of the mean values to their probable errors indicates that the major changes observed in both the red cell and hemoglobin levels are of statistical significance, as shown by the following examples:

Group number	Red blood cells			Hemoglobin		
	Dates of mean values		Ratio	Dates of mean values		Ratio
I	Jan. 10	Jan. 24	3.65	Oct. 24	Jan. 10	2.94
	Apr. 3	June 19	5.44	Apr. 24	June 19	5.24
II	Mar. 29	May. 8	2.96	Mar. 29	Apr. 24	2.73
	Apr. 3	June 12	3.05	Apr. 24	June 5	4.09
III	Sept. 20	Nov. 22	4.53	Sept. 20	Nov. 2	7.00
IV	Nov. 27	June 18	5.38	Nov. 27	June 18	4.80
	Feb. 26	Apr. 30	3.68	Dec. 11	Jan. 22	2.96
V	Dec. 29	Feb. 7	5.40	Jan. 24	Apr. 19	4.09
	Feb. 21	Apr. 26	3.25	Apr. 26	June 14	5.26

A striking feature of these results is the degree of parallelism shown by two groups of animals examined over the same period with respect to the fluctuations of erythrocytes and hemoglobin mean values. The curves in Text-figs. 1 and 2 show that the trend of values for one group of animals is almost always reflected in a similar trend for the other group.

Finally, these observations make it clearly evident that the results obtained on the groups of rabbits examined in one year may not be entirely similar to those obtained on other groups followed for similar periods in another year. In the case of both the erythrocytes and the hemoglobin, the mean values during 1927-28 were generally higher than those of 1928-29. Furthermore, there was a definite trend on the part of both red cell and hemoglobin means toward higher values in the spring and early summer months of the first year which were not observed in the following year. In the fall and early winter months of both years, on the other hand, the red cells tended toward smaller values; this was also the case for the hemoglobin in the second but not in the first year.

CONCLUSIONS

Observations are reported on the consecutive weekly erythrocyte counts and the hemoglobin contents of the peripheral blood in 5 groups of normal rabbits, comprising 45 animals, during a period of 20 months from October, 1927 to July, 1929. The duration of individual group examinations varied from 8 to 35 weeks. The results are analyzed on the basis of the weekly mean values of each group.

On the whole, the erythrocyte values were quite uniform within a narrow range of variation, while the hemoglobin content was comparatively irregular within a wider range of variation. The major changes in the levels of mean values of both the red cells and the hemoglobin, however, were found to be statistically significant.

The directions or trends in the levels of the erythrocyte and hemoglobin mean values did not necessarily move in opposite directions.

The general levels of the erythrocyte and hemoglobin mean values were not identical for two consecutive years, those of 1927-28 being higher than those of 1928-29.

The fluctuations of both red cell and hemoglobin mean values

observed in one group of animals were also usually observed in another group examined during the same months.

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STUDIES IN THE BLOOD CYTOLOGY OF THE RABBIT

III. CONSECUTIVE TOTAL WHITE BLOOD CELL OBSERVATIONS ON GROUPS OF NORMAL RABBITS

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In the preceding paper of this series, experiments dealing with consecutive observations on the peripheral cytology of normal rabbits for prolonged periods of time were described and the results pertaining to the erythrocytes and the hemoglobin were reported (1). The observations were made on 5 groups of male rabbits, 4 of 10 and 1 of 5 animals, during a period extending from October, 1927 to July, 1929. In the present paper, the results relating to the total white cell counts are reported together with a consideration of these values from the standpoint of the total granular leucocytes—the neutrophiles (pseudo-eosinophiles), the basophiles, and the eosinophiles—on the one hand, and the total non-granular cells—the lymphocytes and the monocytes—on the other.

Materials and Methods

Since the conduct of the experiments and the methods employed in analyzing the results obtained have previously been described (1), it will suffice here merely to state that in the great majority of cases the examination of the blood was carried out at weekly intervals. The differential counts which were made by means of the supravital neutral red technic, were based upon counts of 100 cells for each specimen.

The number of examinations on each group of rabbits was: Group I, 35; Group II, 13; Group III, 8; Group IV, 29; Group V, 26. The first 4 groups comprised 10, and the last, 5 animals.

RESULTS

The results on consecutive total white blood cell counts in 5 groups of normal rabbits observed 8 to 35 weeks are given in Tables I to V and

TABLE I

Group I-10 Rabbits. Consecutive Values for Total White Blood Cells and for Total Granular and Non-Granular Cells

Date	Total white blood cells				Granular: Neutrophiles, basophiles, and eosinophiles				Non-granular: Lymphocytes and monocytes			
	Mean values		Standard deviation		Coefficient of variation		Mean values		Standard deviation		Coefficient of variation	
	per cmm.	per cmm.	per cmm.	per cent	per cent	per cmm.	per cmm.	per cmm.	per cmm.	per cmm.	per cent	per cent
1927-28												
Oct. 24*	8825 ± 401	1879	21.29	21.29	5586 ± 261	1224	21.91	3240 ± 243	1138	35.12		
Nov. 1.....	8245 ± 430	2015	24.44	24.44	5293 ± 304	1423	26.89	2953 ± 188	879	29.77		
Nov. 8**	8030 ± 507	2376	29.59	29.59	4948 ± 283	1328	26.84	3082 ± 261	1222	39.65		
Nov. 15.....	8555 ± 275	1288	15.06	15.06	4910 ± 192	901	18.35	3645 ± 132	618	16.95		
Nov. 22.....	9935 ± 601	2816	28.34	28.34	6522 ± 355	1666	25.54	3414 ± 224	1048	30.70		
Nov. 29.....	9685 ± 747	3504	36.18	36.18	5494 ± 578	2710	49.33	4192 ± 265	1241	29.60		
Dec. 6.....	8840 ± 463	2169	24.54	24.54	4987 ± 395	1852	37.14	3854 ± 177	831	21.56		
Dec. 13.....	9130 ± 756	3546	38.84	38.84	4783 ± 393	1841	38.49	4348 ± 469	2198	50.55		
Dec. 20.....	9690 ± 582	2729	28.16	28.16	6251 ± 417	1955	31.27	3439 ± 196	920	26.75		
Dec. 27.....	9400 ± 380	1780	18.94	18.94	5983 ± 258	1209	20.21	3418 ± 200	936	27.38		
Jan. 3.....	9800 ± 469	2200	22.45	22.45	5811 ± 254	1192	20.51	3990 ± 347	1628	40.80		
Jan. 10.....	10240 ± 440	2065	20.17	20.17	5827 ± 338	1586	27.22	4392 ± 255	1195	27.21		
Jan. 17.....	10375 ± 613	2873	27.69	27.69	5899 ± 496	2327	39.45	4454 ± 345	1616	36.28		
Jan. 24.....	10380 ± 481	2253	21.70	21.70	5891 ± 277	1301	22.08	4478 ± 260	1217	27.18		
Jan. 31.....	11081 ± 718	3367	30.39	30.39	6590 ± 520	2439	37.01	4466 ± 335	1571	35.18		
Feb. 7.....	11095 ± 651	3053	27.52	27.52	6477 ± 544	2550	39.37	4601 ± 240	1123	24.41		
Feb. 14.....	10920 ± 671	3146	28.81	28.81	6643 ± 554	2595	39.06	4270 ± 77	362	8.48		
Feb. 21.....	12450 ± 504	2365	19.00	19.00	6545 ± 353	1654	25.27	5888 ± 246	1152	19.57		
Feb. 28.....	11700 ± 913	4281	36.59	36.59	8052 ± 842	3950	49.06	3651 ± 65	303	8.30		
Mar. 6.....	10455 ± 494	2316	22.15	22.15	6390 ± 329	1543	24.15	4051 ± 230	1078	26.61		
Mar. 13.....	9910 ± 657	3079	31.07	31.07	6186 ± 505	2368	38.28	3715 ± 276	1296	34.89		
Mar. 20.....	11270 ± 792	3711	32.93	32.93	7164 ± 760	3561	46.77	3647 ± 170	795	21.80		

Mar. 27.....	10230 ± 499	2338	22.85	6062 ± 372	1742	28.74	4156 ± 235	1100	26.47
Apr. 3.....	10750 ± 620	2908	27.16	6202 ± 429	2013	32.46	4503 ± 397	1859	41.28
Apr. 10.....	12275 ± 679	3183	25.93	7271 ± 464	2174	29.90	4992 ± 221	1038	20.79
Apr. 17.....	11225 ± 543	2547	22.69	6385 ± 379	1779	27.86	4840 ± 255	1195	24.69
Apr. 24.....	10535 ± 718	3367	31.96	6522 ± 561	2629	40.31	4006 ± 315	1475	36.84
May 1.....	11240 ± 570	2670	23.75	6130 ± 303	1432	23.20	5098 ± 301	1411	27.68
May 8.....	11100 ± 726	3404	30.67	6644 ± 481	2255	33.94	4139 ± 309	1448	32.62
May 15.....	11605 ± 504	2364	20.37	6761 ± 388	1819	26.90	4845 ± 199	933	19.26
May 22.....	12085 ± 698	3273	27.08	7217 ± 570	2670	37.00	4869 ± 232	1088	22.35
May 29.....	12035 ± 901	4226	35.11	6443 ± 369	1732	26.88	5447 ± 594	2787	51.17
June 5.....	13590 ± 532	2495	18.36	7445 ± 312	1461	19.62	6144 ± 321	1504	24.18
June 12.....	12380 ± 797	3735	30.17	6656 ± 330	1547	23.24	5725 ± 474	2221	38.79
June 19.....	11455 ± 831	3895	34.00	6973 ± 497	2332	33.44	4482 ± 402	1883	40.90
Mean.....	10585 ± 146	1281	12.10	6269 ± 87	759	12.11	4307 ± 87	763	17.72
Minimum.....	8030			4783			2953		
Maximum.....	13590			8052			6144		

* October 24 and 26.

** November 4 and 9.

TABLE II
Group II—10 Rabbits. Consecutive Values for Total White Blood Cells and for Total Granular and Non-Granular Cells

Date	Total white blood cells			Granular: Neutrophiles, basophiles, and eosinophiles			Non-granular: Lymphocytes and monocytes		
	Mean values	Standard deviation	Coefficient of variation	Mean values	Standard deviation	Coefficient of variation	Mean values	Standard deviation	Coefficient of variation
	per cmm.	per cmm.	per cent	per cmm.	per cmm.	per cent	per cmm.	per cmm.	per cent
1928									
Mar. 29.....	7095 \pm 275	1290	18.18	4300 \pm 257	1206	28.05	2783 \pm 185	866	31.12
Apr. 3.....	8735 \pm 422	1979	22.66	4745 \pm 205	962	20.07	3990 \pm 287	1345	33.71
Apr. 10.....	8445 \pm 534	2504	29.65	4516 \pm 346	1622	35.92	3917 \pm 320	1498	38.24
Apr. 17.....	10150 \pm 531	2487	24.50	5640 \pm 352	1652	29.29	2405 \pm 348	1631	36.20
Apr. 24.....	9350 \pm 525	2462	26.33	5430 \pm 367	1720	31.68	3900 \pm 240	1126	28.72
May 1.....	8040 \pm 315	1477	18.37	4103 \pm 209	978	23.84	3927 \pm 237	1109	28.17
May 8.....	9085 \pm 394	1845	20.31	5093 \pm 299	1401	27.51	3992 \pm 233	1092	27.35
May 15.....	7610 \pm 579	2716	35.69	4285 \pm 390	1829	42.68	3325 \pm 276	1293	38.89
May 22.....	11120 \pm 449	2107	18.95	6132 \pm 351	1645	26.83	4988 \pm 279	1308	26.22
May 29.....	9460 \pm 702	3289	34.77	4847 \pm 313	1465	30.22	4601 \pm 416	1949	42.36
June 5.....	10230 \pm 445	2088	20.41	5570 \pm 317	1485	26.66	4660 \pm 283	1325	28.43
June 12.....	9030 \pm 557	2611	28.91	4772 \pm 254	1190	25.20	4229 \pm 323	1514	35.22
June 19.....	10355 \pm 375	1756	16.96	5663 \pm 267	1250	22.07	4692 \pm 234	1097	23.38
Mean.....	9131 \pm 208	1114	12.20	5007 \pm 114	609	12.16	3958 \pm 135	722	18.24
Minimum.....	7095			4103			2405		
Maximum.....	11120			6132			4988		

TABLE III
Group III—10 Rabbits. Consecutive Values for Total White Blood Cells and for Total Granular and Non-Granular Cells

Date	Total white blood cells			Granular: Neutrophiles, basophiles, and eosinophiles			Non-granular: Lymphocytes and monocytes		
	Mean values	Standard deviation	Coefficient of variation	Mean values	Standard deviation	Coefficient of variation	Mean values	Standard deviation	Coefficient of variation
	per cmm.	per cmm.	per cent	per cmm.	per cmm.	per cent	per cmm.	per cmm.	per cent
1923									
Sept. 20.....	8040 \pm 672	3152	39.20	5337 \pm 355	1602	31.02	2683 \pm 281	1319	49.16
Sept. 28.....	7030 \pm 474	2223	31.62	3796 \pm 176	826	21.76	3234 \pm 292	1368	42.30
Oct. 10.....	7715 \pm 487	2282	29.58	4779 \pm 427	2000	41.68	2916 \pm 99	464	15.91
Oct. 19.....	6985 \pm 381	1785	25.55	3675 \pm 119	553	15.05	3310 \pm 207	969	29.27
Nov. 2.....	7560 \pm 337	1581	20.91	4754 \pm 204	955	20.09	2806 \pm 101	473	16.86
Nov. 9.....	7885 \pm 297	1393	17.67	4713 \pm 292	1370	29.07	3173 \pm 113	783	24.68
Nov. 16.....	6595 \pm 270	1264	19.17	3940 \pm 144	674	17.11	2655 \pm 176	824	31.04
Nov. 22.....	7165 \pm 314	1473	20.56	3922 \pm 168	788	20.09	3243 \pm 196	921	28.40
Mean.....	7372 \pm 112	471	6.39	4365 \pm 135	566	12.97	3003 \pm 60	251	8.36
Minimum.....	6985			3675			2655		
Maximum.....	8040			5337			3310		

TABLE IV

Group IV—10 Rabbits. Consecutive Values for Total White Blood Cells and for Total Granular and Non-Granular Cells

Date	Total white blood cells			Granular: Neutrophils, basophiles, and eosinophiles			Non-granular: Lymphocytes and monocytes		
	Mean values	Standard deviation	Coefficient of variation	Mean values	Standard deviation	Coefficient of variation	Mean values	Standard deviation	Coefficient of variation
	per cmm.	per cmm.	per cent	per cmm.	per cmm.	per cent	per cmm.	per cmm.	per cent
1928-29									
Nov. 27.....	7755 ± 201	1605	20.69	4593 ± 265	1243	27.07	3162 ± 195	918	29.03
Dec. 4.....	7665 ± 357	1673	21.82	4453 ± 308	1445	32.44	3212 ± 162	762	23.72
Dec. 11.....	7245 ± 438	2054	28.34	4467 ± 308	1446	32.37	2771 ± 216	1012	36.51
Dec. 18.....	6660 ± 430	2014	30.23	3670 ± 198	926	25.23	2958 ± 291	1365	46.13
Dec. 26.....	6720 ± 357	1673	24.88	4192 ± 281	1319	31.46	2522 ± 143	679	26.91
Jan. 2.....	6340 ± 237	1111	17.52	4320 ± 209	979	22.66	2019 ± 81	390	19.32
Jan. 8.....	6125 ± 403	1890	30.86	3595 ± 326	1527	42.48	2530 ± 166	790	31.21
Jan. 15.....	6620 ± 270	1265	19.10	4087 ± 221	1035	25.31	2518 ± 96	458	18.20
Jan. 22.....	6645 ± 431	2021	30.41	4102 ± 274	1283	31.28	2543 ± 201	953	37.47
Jan. 29.....	8570 ± 462	2166	25.27	5510 ± 322	1509	27.37	3061 ± 179	851	27.80
Feb. 5.....	6535 ± 412	1932	29.56	4116 ± 325	1526	37.07	2419 ± 186	884	36.53
Feb. 13.....	8840 ± 550	2579	29.17	5978 ± 391	1833	30.66	2862 ± 207	980	34.24
Feb. 19.....	9890 ± 352	1648	16.66	7050 ± 319	1495	21.19	2800 ± 159	757	27.04
Feb. 26.....	9515 ± 418	1969	20.59	6364 ± 359	1683	26.44	3104 ± 141	673	21.67
Mar. 12.....	7785 ± 261	1224	15.71	4731 ± 186	873	18.44	3013 ± 195	922	30.61
Mar. 19.....	9305 ± 484	2267	24.36	6259 ± 423	1982	31.66	2046 ± 175	829	27.20
Mar. 26.....	8875 ± 501	2349	26.46	5926 ± 371	1741	29.37	2925 ± 237	1119	38.26
Apr. 2.....	8475 ± 520	2436	28.74	6058 ± 398	1865	30.78	2417 ± 218	1032	42.69
Apr. 9.....	9365 ± 343	1610	17.19	5686 ± 146	685	12.04	3596 ± 240	1136	46.98
Apr. 16.....	9360 ± 470	2204	23.54	5886 ± 326	1527	25.94	3474 ± 252	1291	37.16
Apr. 23.....	10185 ± 440	2062	20.24	6246 ± 254	1193	19.10	3887 ± 249	1276	32.82
Apr. 30 ...	8840 ± 347	1625	18.38	5912 ± 244	1144	19.36	2920 ± 214	1113	38.09

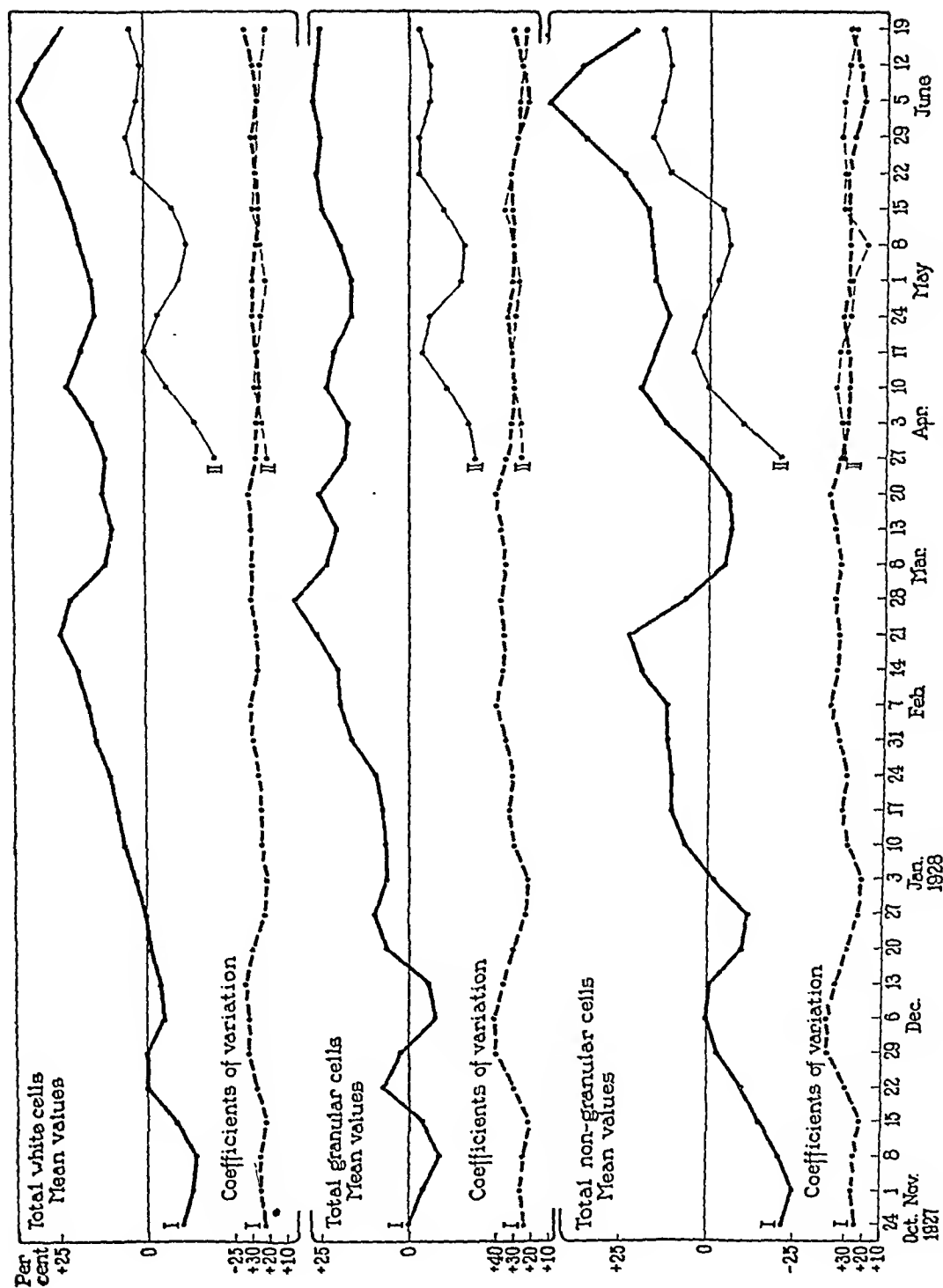
May 7.....	9375 ± 259	1212	12.92	6066 ± 215	1010	16.64	3273 ± 133	732	22.37
May 14.....	8067 ± 342	1523	18.87	4901 ± 232	1086	22.14	3136 ± 134	739	23.57
May 21.....	7478 ± 275	1223	16.35	5241 ± 236	1106	21.11	2237 ± 81	490	21.89
May 28.....	8439 ± 351	1560	18.48	5421 ± 273	1278	23.67	3002 ± 127	707	23.54
June 4.....	7183 ± 313	1392	19.38	4545 ± 269	1260	27.71	2638 ± 134	739	28.00
June 11.....	7006 ± 350	1557	22.22	4577 ± 237	1111	24.27	2429 ± 96	562	23.12
June 18.....	7031 ± 265	1113	15.83	3883 ± 162	758	19.53	3139 ± 192	1011	32.20
Mean.....	7996 ± 148	1183	14.79	5098 ± 130	1039	20.38	2883 ± 53	411	14.30
Minimum.....	6125			3595			2019		
Maximum.....	10185			7050			3887		

TABLE V

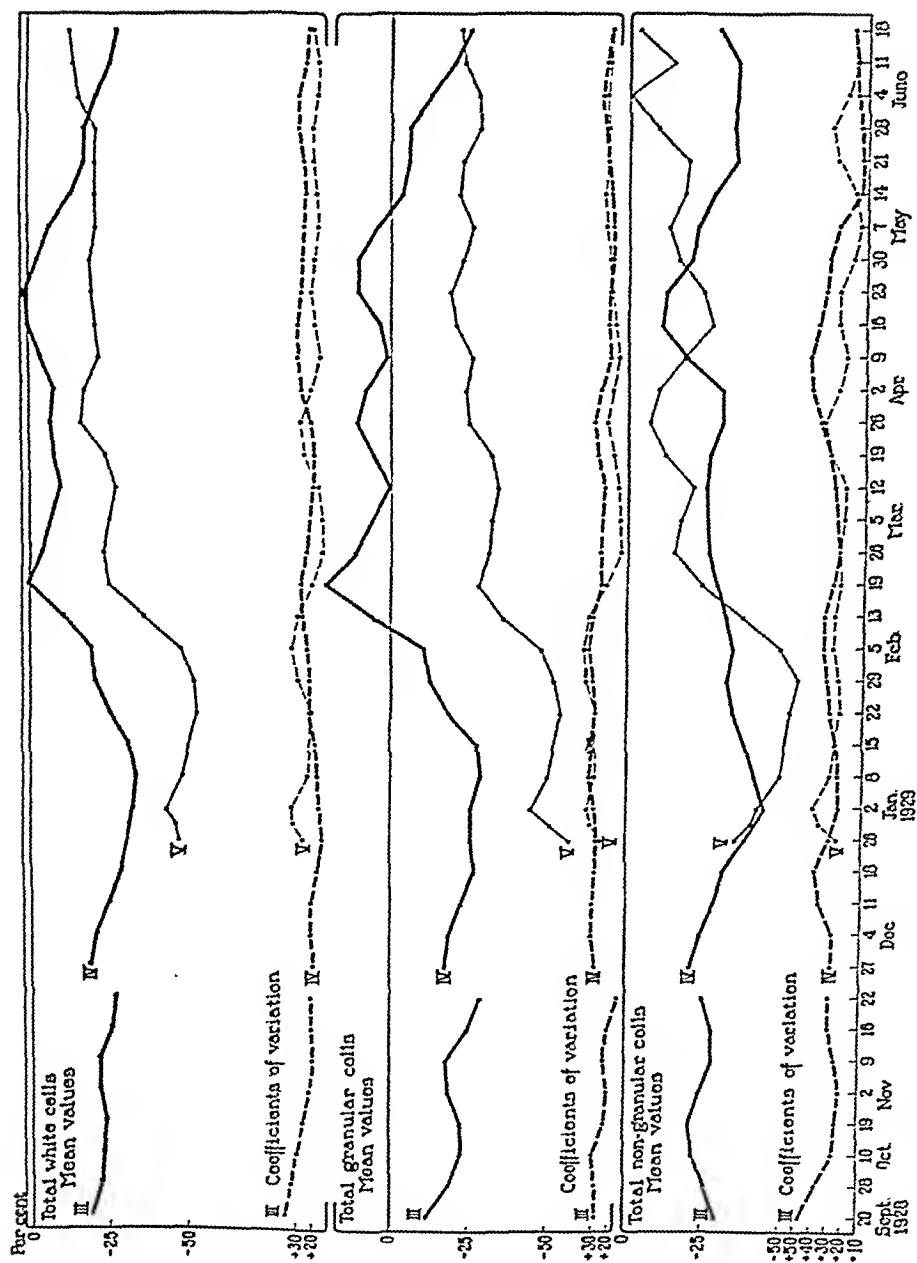
Group V—5 Rabbits. Consecutive Values for Total White Blood Cells and for Total Granular and Non-Granular Cells

Date	Total white blood cells			Granular: Neutrophiles, basophiles, and eosinophiles			Non-granular: Lymphocytes and monocytes		
	Mean values	Standard deviation	Coefficient of variation	Mean values	Standard deviation	Coefficient of variation	Mean values	Standard deviation	Coefficient of variation
	per cmm.	per cmm.	per cent	per cmm.	per cmm.	per cent	per cmm.	per cmm.	per cent
1928-29									
Dec. 29	5310 ± 338	1119	21.07	2369 ± 178	591	24.95	2941 ± 177	586	19.93
Dec. 31	4410 ± 441	1463	33.17	2390 ± 246	817	34.18	2020 ± 222	736	36.44
Jan. 3	6260 ± 714	2367	37.81	3555 ± 371	1231	34.63	2697 ± 421	1396	51.76
Jan. 10	4510 ± 248	822	18.23	2514 ± 255	844	33.57	1996 ± 121	402	20.14
Jan. 24	4110 ± 138	458	11.14	2431 ± 131	433	17.81	1679 ± 150	496	29.54
Jan. 31	4820 ± 549	1820	37.76	2812 ± 404	1340	47.65	2008 ± 125	414	20.62
Feb. 7	4240 ± 322	1066	25.14	2451 ± 186	617	25.17	1789 ± 161	532	29.74
Feb. 15	6400 ± 757	2509	39.20	3648 ± 506	1677	45.97	2752 ± 271	898	32.63
Feb. 21	7260 ± 219	725	9.99	4294 ± 200	662	15.42	2966 ± 122	405	13.65
Mar. 1	7210 ± 347	1149	15.94	3547 ± 115	380	10.71	3663 ± 390	1294	35.33
Mar. 8	7380 ± 203	672	9.11	3921 ± 168	556	14.18	3459 ± 179	593	17.14
Mar. 15	6510 ± 335	1111	17.07	3562 ± 163	540	15.16	2948 ± 171	567	19.23
Mar. 22	7010 ± 459	1521	21.70	3594 ± 166	550	15.30	3426 ± 311	1031	30.09
Mar. 29	8320 ± 855	2833	34.05	4323 ± 361	1195	27.64	3997 ± 522	1730	43.28
Apr. 5	8070 ± 430	1425	17.66	4360 ± 238	789	18.10	3710 ± 262	867	23.37
Apr. 12	6960 ± 209	692	9.94	3657 ± 138	456	12.47	3303 ± 138	456	13.81
Apr. 19	7600 ± 386	1280	16.84	4734 ± 257	852	18.00	2866 ± 271	898	31.33
Apr. 26	7220 ± 522	1730	23.96	4341 ± 281	932	21.47	2879 ± 255	845	29.35
May 3	8070 ± 401	1328	16.46	4355 ± 255	844	19.38	3715 ± 172	569	15.32
May 10	7100 ± 327	1085	15.28	3837 ± 274	908	23.66	3263 ± 127	422	12.93
May 17	7760 ± 402	1333	17.18	4350 ± 339	1123	25.82	3410 ± 159	528	15.48
May 24	7130 ± 317	1052	14.75	4364 ± 275	913	20.92	2767 ± 217	718	25.95

May 31.....	7990 ± 657	2179	27.27	3865 ± 265	877	22.69	4125 ± 570	1888	45.77
June 7.....	7510 ± 150	497	6.62	3717 ± 271	897	24.13	3793 ± 144	477	12.58
June 14.....	8560 ± 461	1528	17.85	4624 ± 297	986	21.32	3936 ± 194	642	16.31
June 21.....	8060 ± 460	1526	18.93	4095 ± 264	876	21.39	3965 ± 237	785	19.80
Mean.....	6761 ± 177	1334	19.73	3681 ± 97	730	19.83	3087 ± 95	718	23.26
Minimum.....	4110			2369			1679		
Maximum.....	8560			4734			3997		



TEXT-FIG. 1. Mean values for consecutive total white blood, granular and non-granular cell determinations as percentage deviations from standard values. 1927-28.



TEXT-FIG. 2. Mean values for consecutive total white blood, granular and non-granular cell determinations as percentage deviations from standard values, 1928-29.

in Text-figs. 1 and 2. The results have further been considered from the standpoint of the total granular and the total non-granular cells (Tables I to V; Text-figs. 1 and 2). In all cases, the observations are presented in the form of weekly group means together with the probable errors of the means, the standard deviations, and the coefficients of variation. The series of curves in Text-figs. 1 and 2 representing the smoothed means are drawn in the form of the percentage deviations of these values from standard values (1, 2). The standard values used are as follows: Total white cells, 9560 per cubic millimeter; total granular cells, 5505 per cubic millimeter; total non-granular cells, 4050 per cubic millimeter. Other curves illustrate the smoothed values of the coefficients of variation of the group means.

DISCUSSION AND SUMMARY

In discussing the results on the consecutive total white cell counts of 5 groups of normal rabbits studied over long periods of time, it will be convenient to consider the observations of each group in chronological order. The results will then be taken up from the standpoint of the granular and the non-granular cells.

Group I was examined from October 24, 1927 to June 19, 1928. During the 9 months of the experiment, it was found that the mean total white cell counts were considerably increased as determined by weekly observations (Table I). The character and extent of this change is shown in the curve (Text-fig. 1) which represents the percentage deviations of the smoothed weekly means from a standard value of 9560 cells per cubic millimeter.

From an initial level of 10 per cent below the standard value in October and November, 1927, there was a fairly regular increase to 25 per cent above it by the end of February; in March and April, somewhat lower levels were observed, but in May there was another rise to 35 per cent, followed in June by slightly lower counts. It should be noted that the curve of the smoothed coefficients of variation of the means (Text-fig. 1) shows only slight irregularities throughout its course, thus indicating that the alterations of the mean values were generally characteristic of the group as a whole.

In the case of Group II (Table II) which was examined from March 29 to June 19, 1928, a similar increase of the total white cells was observed.

The level of mean values was lower than that of Group I for the same period, but the general character of the increase resembled in a striking manner that of the former group (Text-fig. 1). The curve of the smoothed coefficients of variation of the weekly means of Group II is quite uniform and occupies the same level as that of Group I.

The mean white cell values of Group III, followed from September 20 to November 22, 1928 (Table III) were lower than those of Group I or of Group II and in addition, differed from these first groups in showing only minor fluctuations.

The curve illustrating these findings (Text-fig. 2) presents few irregularities and is, in fact, almost a straight line at the 20 to 25 per cent level below the standard value. The first part of the curve of the smoothed coefficients of variation of the weekly means (Text-fig. 2) is slightly higher than those of Groups I and II but the second half is entirely comparable.

The fourth group of rabbits was followed from November 27, 1928 to June 18, 1929 (Table IV). In general, the level of the white cell means (Text-fig. 2) was similar to that of Group III and lower than that of Group I examined the previous year. It was found, as was the case with Groups I and II, that these values became considerably increased.

In November and December, the lowest counts of the group were observed; in January and February, higher values prevailed; during March and April, slightly lower levels were fairly well maintained with a second increase at the end of April which in turn, was followed by a steady decline to values comparable to those observed at the beginning of the experiment. The coefficients of variation of the white cell means of Group IV were of the same general order of magnitude as those of the first 3 groups, but they were somewhat more irregular as is brought out by a comparison of the respective curves of the smoothed values shown in Text-figs. 1 and 2.

In comparing these changes with those of Group I observed during a similar period of the previous year (Text-figs. 1 and 2), it will be noted that as far as form and direction are concerned, the curves for these groups bear a general resemblance to each other up to May and June. In the case of Group I, higher levels continued to be maintained during these months although the final direction of the curve is downward; in the case of Group IV, the downward trend which began in April was continued throughout June.

The observations on Group V extended from December 19, 1928 to June 21, 1929 (Table V); this series comprised 5 as contrasted with 10 rabbits in each of the other groups. The level of the white cell means was consistently lower than that of any other group, but as was found in Groups I, II, and IV, the values became increased.

As far as trends are concerned, the first half of the Group V curve, representing the observations of December, January, February, and March, shows a striking similarity to the curve of Group IV for the same period; the latter part, however, differs in that the March level continues to be fairly constantly maintained to the end of the experiment while the curve of Group IV first rises (April) and then falls (May and June), so that eventually the two curves cross each other. The coefficients of variation of the weekly means of Group V (Text-fig. 2) are, as was the case with Group IV, more irregular than those of Groups I, II, and III, but they are of the same general order of magnitude as the others.

Turning now to a consideration of the white cells from the standpoint of their division into granular leucocytes and non-granular lymphocytes and monocytes (Table I), it will be seen by referring to Text-fig. 1, that in the case of Group I, both the granular and non-granular cells participated in the increase of the mean total white count. As far as general contour is concerned, the curves representing the means of these cells are similar to the curve of the total white cell means, but they are more irregular and their fluctuations are more pronounced, especially in the case of the non-granular cells. For the most part, the shifts in the trends of these curves are similar in direction, and not infrequently, a change in the non-granular curve precedes that of the granular curve. At the time of the pronounced drop in the total white cell means in March which occurred after a prolonged increase in values, it will be noted that there was a marked decrease in the non-granular means which was relatively much greater than the decrease of the granular cell means. In like manner, the participation of the non-granular cells in the prolonged rise of the total white cell curve during May and June was relatively much more pronounced than that of the granular leucocytes. With respect to the coefficients of variation of these means, the curves illustrating their smoothed values also show more irregularities than that of the total white means but all are of the same general order of magnitude.

In the case of Group II (Table II) the granular and non-granular cell means were found to have the same general relationship to each other and to the total white

cell means as in Group I, as is illustrated by the curves in Text-fig. 1. And the coefficients of variations of these means were likewise similar to those of Group I (Table II, Text-fig. 1).

The observations on Group III (Table III) differ from those of Groups I and II in that the trends of the slight variations of the granular and non-granular means are consistently in opposite directions as is shown by the curves of Text-fig. 2. The curve representing the total white cell means is practically a straight line. The curves of the smoothed coefficients of variation for the granular and non-granular means show no significant differences from those of Groups I and II.

In the case of Group IV (Table IV), the general character of the trends of the granular and non-granular cell means with respect to each other and to the total white cell means resembled those of Groups I and II (Text-fig. 2) but in certain features, the results were different.

Thus, there are more irregularities in the curve representing the granular cells than in the curve of the non-granular cells and the fluctuations are more pronounced. Furthermore, the increase in the total mean counts during January and February and the decreasing values in May and June were participated in by a greater relative increase and decrease of the granular than of the non-granular means. The coefficients of variation of both granular and non-granular means of Group IV (Table IV, Text-fig. 2) were of the same order of magnitude as those of the previous groups.

The results of Group V (Table V) with respect to the consecutive granular and non-granular cell means resemble on the whole those of Groups I and II rather than those of Group IV, as may be seen by comparing the curves in Text-figs. 1 and 2.

While the general trend of both the granular and non-granular curves is definitely upward, the rise of the non-granular curve is more pronounced than that of the granular, indicating a greater relative increase in the mean number of non-granular cells. This feature was found in Groups I and II but the reverse occurred in Group IV. In addition, the non-granular cell curve of Group V is somewhat more irregular and the fluctuations are more marked as was also the case with Groups I and II but not with Group IV. The coefficients of variations of the granular and non-granular cell means of Group V (Table V) are of the same order of magnitude as those of the other groups (Text-fig. 2).

It should be specially noted that in the groups observed for the longest periods, the mean values of the total white cells became considerably increased. In Groups I, II, and V, followed 35, 13, and 26

weeks respectively, the values during the latter part of the experiments were all higher than those of the first weeks. Both the granular and non-granular cell means participated in this change, but from a relative standpoint, the latter values showed the greater alteration. In the case of Group IV, followed 29 weeks, a similar increase of the total white cells occurred although it was not sustained during the last 8 weeks; the granular cell means of this group showed a greater relative change than those of the non-granular cells.

In connection with these findings of augmented white cell counts over periods of several months, it might be thought that the increasing age of the rabbits was a factor of some significance; at the beginning of these experiments, the animals were considered to be 6 to 8 months old. According to the recent observations of Cheng (5), however, the numbers of leucocytes in the blood stream of rabbits 9 to 16 months of age, although fluctuating at a high level, are not increased. The question of intercurrent infections such as snuffles, as a factor in the production of increased white counts will be considered in connection with the results on the individual classes of white cells (3, 4).

The significance to be attached to the major changes in the levels of the total white, the granular, and the non-granular cell means over periods of time is indicated by the following examples of the ratios of the differences of various mean values to their probable errors.

Group Number	Total white cells			Granular cells			Non-granular cells		
	Dates of mean values		Ratio	Dates of mean values		Ratio	Dates of mean values		Ratio
I	Oct. 24	June 19	2.85	Oct. 24	June 19	2.47	Oct. 24	June 19	2.64
	Mar. 13	June 5	4.36	Dec. 6	Feb. 28	3.30	Feb. 21	Mar. 20	7.52
II	Mar. 29	June 19	7.01	Mar. 29	June 19	3.67	Mar. 29	June 19	6.61
III	Sept. 20	Nov. 16	2.00	Sept. 20	Nov. 22	3.60	Sept. 20	June 19	1.64
IV	Nov. 27	Jan. 2	4.55	Nov. 27	Feb. 19	8.33	Nov. 27	Jan. 2	5.20
	Apr. 9	June 18	5.39	Feb. 19	June 18	8.75	Jan. 2	Apr. 23	7.13
V	Dec. 29	June 21	4.82	Dec. 29	June 21	5.43	Dec. 29	Jan. 24	5.44
	Jan. 24	Apr. 5	8.76	Jan. 4	Apr. 5	7.09	Jan. 24	June 7	10.16

As was the case with the erythrocyte and hemoglobin mean values of these experiments (1), the general level of the total white cell means was higher in the groups of rabbits examined during 1927-28 than in those examined in 1928-29. This was also true of both the granular and the non-granular cells but was less pronounced in the former than in the latter class.

The present results are analogous to those of the erythrocytes and hemoglobin (1) in still another respect. It has been shown that as far as general direction or trend of the total white cell means is concerned and in the case of many individual fluctuations as well, two groups of rabbits examined during the same months behave in a similar manner. In the case of the granular and non-granular divisions of white cells, however, the resemblance is not always as striking. With the granular and non-granular cells of Groups I and II and with the granular cells of Groups IV and V, the similarity was quite pronounced, but with the non-granular cells of Groups IV and V, the parallelism was less marked.

In most cases, an alteration in the trend of the granular cell means of a group was accompanied by a movement of the non-granular cell values in the same direction but in certain instances, a change in the opposite direction was observed.

The period of most frequent point to point fluctuation of the total white cell and of the granular and non-granular cell means of the 4 groups examined for several months occurred in February, March, and April of both years (1928 and 1929). A similar but less pronounced phase of irregularity affecting chiefly the total white and the granular cells was observed in October and November of 1927 but not in the fall months of 1928.

CONCLUSIONS

Consecutive weekly observations on the total white cell count of the peripheral blood were made on 5 groups of normal rabbits, a total of 45 animals, during a period of 20 months from October, 1927 to July, 1929. The duration of individual group examinations varied from 8 to 35 weeks.

In the case of 4 groups followed 13 to 35 weeks, the general trend of the total white cell means was toward increasing values; with the

group followed 8 weeks, the means were maintained at a constant level.

The changes in the levels of the granular cell means were usually accompanied by changes in a similar direction of the non-granular cell means. In the case of 3 of the 4 groups followed for the longest periods, the greatest relative alterations occurred in the non-granular cells.

The fluctuations in the mean values of the total white cells and of the granular and the non-granular cells which were observed in one group of rabbits, were also generally observed in another group examined during the same months.

The period of greatest irregularity in the mean values of the total white cell means and of the granular and the non-granular cell means as well, occurred during the late winter and spring months of both years.

The general level of the total white cell mean values in the groups examined during 1927-28 was higher than that of the groups observed during 1928-29. A similar difference was found in the granular and non-granular mean values but it was somewhat less marked in the case of the granular cells.

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TOOTH GROWTH IN EXPERIMENTAL SCURVY

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PLATES 1 TO 4

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The lesions in the incisor teeth of scorbutic guinea pigs have been repeatedly studied since their recognition by Jackson and Moore (1). This paper is concerned with an associated phenomenon, namely, the rate of tooth growth in scurvy and partial scorbutic states as well as the effect of stress upon growth.

Through the work of Zilva (2), lesions of the roots of the incisor teeth of guinea pigs have been established as the first expression of a deficiency of Vitamin C. Höjer utilized this fact in testing the antiscorbutic value of foodstuffs, the minimal protective dose being the smallest amount of the antiscorbutic which would completely protect the teeth of guinea pigs throughout a 20 day period (3). As Eddy (4) has shown, the minimal protective dose by this method is much greater than by the more generally employed test (the Sherman method), which employs the weight curve of the animal fortified by certain clinical observations as pain over joints, presence of hemorrhages, etc. These and other related facts have been recently emphasized by Hanke (5), and others, and the rôle of Vitamin C in tooth development and health has become a subject of considerable interest.

Rate of Tooth Growth

We have found one reference to the influence of diet on rate of tooth growth. Orban (6) noted the rate of growth of rats' teeth when starch alone was fed and found it less rapid than when wheat, meat and greens were given. Feeding wheat and cod liver oil, the growth rate obtained approximated the rate secured with wheat, meat and greens. The addition of lemon juice did not increase this rate.

In our studies rate of growth was measured by clipping the exposed portion of one of the lower incisor teeth every fifth day. A pocket finger nail clip was

found to be the most satisfactory instrument for the purpose since it was of convenient size, preserved the whole of the fragment intact, and required no prying force, hence producing the minimum of trauma to the root of the tooth. If, after repeated clipping, the tooth became very fragile and brittle it was first cut three-fourths through with a very thin carborundum disk used on a dental engine, and then clipped. By folding the pig's ears forward and keeping the disk wet the operation does not excite the animals. The length of the fragment was measured with vernier calipers. Results have been expressed as averages in millimeters per day. The animals used in the experiment were observed for periods of varying length, the shortest being 20 days and the longest period 90 days.

Since the normal rate of tooth growth was unknown, this was first established, using animals from 4 to 10 weeks of age, and from 110 gm. to 600 gm. In the smaller animals the rate of tooth growth was slightly less rapid than in the larger, but this factor was too small to affect the result of the feeding experiments.

The basal diet used was the Sherman-La Mer (7) to which 1 per cent of cod liver oil had been added. During the period of test the animals were scored by the Sherman method for degree of scurvy and were weighed every fifth day. At the end of the experiment the animals were killed and dissected. The lower jaws, ribs and extremities were removed, fixed, and studied histologically. The incisor roots were sectioned at various levels, one section regularly being removed at the level of the first molar for comparison with Höjer's standards for the estimation of degree of scurvy.

Table 1 summarizes the results obtained by varying amounts of orange juice, tomato juice and turnip greens as the sole source of Vitamin C in combination with Sherman-La Mer basal diet. The group on green vegetables represents the maximum rate of tooth growth and the animals were free of scurvy.¹ In this group the rate of tooth growth was 0.850 mm. daily in contrast to the minimal rate in the control animals of 0.306 mm. daily. In every series the increase in Vitamin C source stimulated growth rate.

Examination of the animals on these diets revealed certain collateral data of value which can be summarized as follows:

1. Animals given basal diet plus 5 cc. of orange juice daily, showed a rate of tooth growth of 0.750 ± 0.002 mm. per day. Slight but definite scorbutic lesions were present in the incisor roots though the long bones and ribs were free of scorbutic changes. The Sherman score showed no scurvy. 5 cc. was therefore not quite adequate for complete protection. 1 cc. of orange juice produced a growth rate of

¹ Greens are notably richer in Vitamin A than fruit juices and it is possible the high maximum with greens may owe something to this factor.

TABLE 1
The Effect of Variation in Amount of Vitamin C Source on Guinea Pig Tooth Regeneration

A	Vitamin source	Amount fed daily		No. animals	Days on experiment	Tooth growth mm. per day	Weight gains	Diagnosis by Sherman score	Diagnosis by Hoffer method
		Basal diet only	dict						
B	Controls			10	20 days	0.306±.0003	-58 gm.	Acute scurvy	
	Orange juice	1 cc.		3	20 "	0.450±.002	+40 "	Mild scurvy	0.3
	" "	5 "		6	20 "	0.750±.002	+55 "	No scurvy	0.9
	" "	0.5 "		3	90 "	0.350±.002	+180 "	Moderate scurvy	
	" "	1 "		2	90 "	0.422±.002	+230 "	Mild scurvy	
C	" "	5 "		2	90 "	0.750±.002	+255 "	No scurvy	
	Tomato juice	2 gm.		3	20 "	0.505±.003	+20 "	Moderate scurvy	0.3-0.8**
	" "	4 "		3	20 "	0.580±.0006	+55 "	No scurvy	0.3
	" "	8 "		3	20 "	0.623±.0006	+70 "	No scurvy	0.8
	" "	10 "		6	20 "	0.700±.0005	+68 "	No scurvy	0.8
D	Turnip greens	2 "		2	20 "	0.565±.0002	+57 "	Mild scurvy	0.3
	" "	4 "		2	20 "	0.640±.0002	+72 "	No scurvy	0.4
	" "	6 "		2	20 "	0.735±.0002	+84 "	No scurvy	0.8
E	Mixed green vegetables*	30 "		3	20 "	0.850±.002	+75 "	No scurvy	1.0

* Mixture included spinach, lettuce and carrots.

** An indefinite type of reaction.

0.450 ± 0.002 mm. to 0.422 ± 0.002 mm. per day. Pronounced scurvy was found in the teeth but none in the long bones or costo-chondral junctions. The Sherman score was "mild scurvy."

Animals on 0.5 cc. of orange juice showed a rate of growth of 0.371 ± 0.005 mm. to 0.350 ± 0.002 mm. per day. Advanced scurvy was present in the teeth and slight changes in the ribs. The Sherman scoring was "moderate scurvy."

Similar results were secured with graded doses of tomato juice and turnip greens. The results are to be found in the table.

2. Animals given basal diet alone showed an average rate of tooth growth of 0.306 ± 0.0003 mm. per day during the first 15 to 20 days but at the end of that period growth ceased completely. However, after complete cessation of growth, the administration of 5 cc. of orange juice daily (commencing on the twentieth day) after a period of lag of 5 days, restored practically the normal rate of tooth growth. Also, so long as the diet afforded growth factor the rate remained constant for any given diet whether the period was 20 or 90 days.

Influence of Stress on Scorbutic Lesions

In an earlier description of the changes encountered in the skeletal muscles of scorbutic guinea pigs (8), it was shown by one of the authors of this paper that exercise and stress determined largely the location and degree of the lesions. It was natural, therefore, to subject the teeth to similar tests since the anatomical lesions of scurvy probably all have this factor in common. As an amputated tooth would be subject to less stress in gnawing than its fellow of normal length, a comparison of the roots of the two teeth should be significant. For this reason in the animals of the series reported in Table 1, and in a number of others in which one incisor had been amputated, a comparison of the histological changes was made by preparing sections through both teeth just anterior to the separation of the mandibles.

The pulp of the incisors of guinea pigs is persistent, and growth, therefore, continuous throughout life. The pulp is roughly shaped like an elongated cone and covered with a surface of tall, slender and parallel odontoblasts (Fig. 2). Growth occurs through the elaboration, on the surface of this cone, of dentin, and, where exposed, enamel.

If a pig is deprived of antiscorbutic substance changes occur in the pulp. By the fifth day of complete deprivation the odontoblasts become shorter and blunter and lose their regularity of parallelism (Fig. 4). In place of dentin they form a semisolid material (Fig. 6) easily converted into dentin within a day by giving antiscorbutic substances, a phase carefully demonstrated by Wolbach and Howe (9).

If complete deprivation persists, the odontoblasts continue to regress and we have seen in the third week root pulp in which the parenchymal cells have left the dentin, developed into spindle and stellate shapes, and resembled fibroblasts (Fig. 5). Wolbach and Howe reported a similar change in the long bones. Death occurs regularly at this time and later stages have not been seen.

If the diet contains even small amounts of Vitamin C, a complete regression of odontoblasts does not occur and instead of fibroblasts the cells come to resemble osteoblasts, forming an intercellular matrix similar to bone which develops within the pulp until, in late stages, the entire cavity becomes filled with this so-called "osteodentin."

If the tooth has been clipped, the evidence of scurvy, both in amount of "osteodentin" and in the character of the cells will be less pronounced than in its fellow (Fig. 1). This has been consistently true of all the cases examined. In animals on a deficient diet for very short periods the amputated tusk may show a root of nearly normal appearance at a time when scurvy is evident in its fellow.

DISCUSSION

These findings and the evidence of the influence of stress on the lesions, indicate that the scorbutic process is characterized by an inability of certain highly differentiated cells to form the intercellular substances natural to them. In cases of partial deficiency an inferior, substitute material may be formed, less highly differentiated than dentin (bone), but when deprivation is complete the cells dedifferentiate still further and form a still more primitive tissue, namely, fibrous tissue. This is similar to what occurs in the long bones and costo-chondral junction where the osteoblasts, appearing unable to form bone matrix, become fibroblasts.

The reaction is increased by demands made on the structure and the lesion constitutes a faulty reaction to stress in the absence of a necessary material.

CONCLUSIONS

1. The incisor teeth of guinea pigs have a constant rate of growth in health.
2. Deprivation of Vitamin C causes the teeth to cease growing. Readministration of the vitamin restores the growth.
3. Administration of small amounts of antiscorbutic substance results in rates of growth roughly proportional to dosage.
4. Under standard experimental conditions used in the testing of foodstuffs for antiscorbutic value, the rate of tooth growth would appear to be a precise indication of the degree of scurvy, being more delicate than the Sherman score, and more constant as well as more simple, than the Höjer method.
5. Stress in terms of usage appears to exaggerate the scorbutic lesions in the teeth.

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EXPLANATION OF PLATES

PLATE 1

FIG. 1. Low power microphotograph of incisor teeth showing differences in structure between amputated (A) and natural (N) teeth in scurvy. The line of odontoblasts (O) is heavier and is formed of longer, more parallel cells in the amputated tooth and the imperfect dentin (N) is narrower.

The difference in breadth of the original dentin is due to the more rapid growth of the amputated tooth and is not related to the scorbutic process.

PLATE 2

FIG. 2. Highly magnified odontoblast line in normal guinea pig tooth. The odontoblasts are slender, parallel and long. The dentin canals are clearly shown.

FIG. 3. Same magnification as Fig. 2. The animal had been given basal diet

plus 3 cc. orange juice daily for 20 days. The cells are shorter and less regular. Near four o'clock an island of imperfect dentin is being formed within the pulp itself by migrated odontoblasts.

PLATE 3

Magnifications same as in Figs. 2 and 3.

FIG. 4. Line of odontoblasts after 5 days on a diet completely deficient in antiscorbutic substance.

FIG. 5. In this animal, which had been for 21 days without antiscorbutic substance the odontoblasts have lost their natural appearance and resemble fibroblasts.

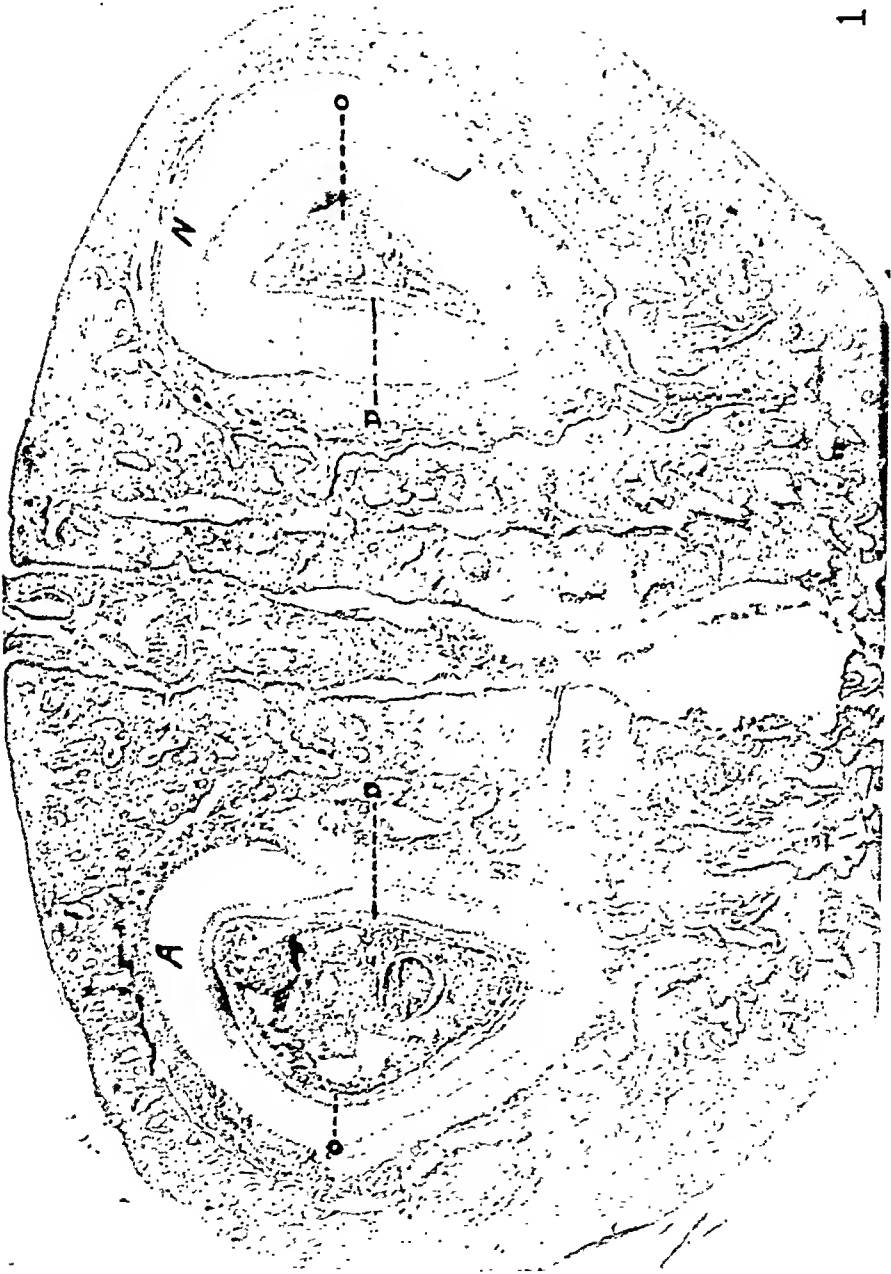
PLATE 4

Magnifications same as in Figs. 2 to 5.

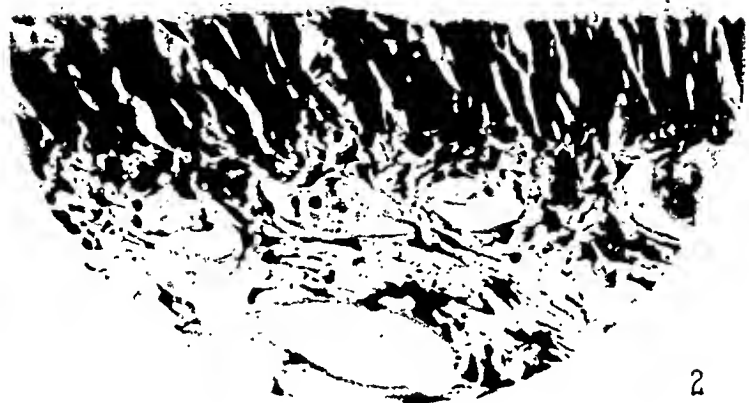
FIG. 6. Illustration of recovery of function by odontoblasts. The animal had been on a Vitamin C-free diet for 10 days and was then given orange juice for 5 days. The cells have become more orderly and a zone of fresh pre-dentin has been formed.

FIG. 7. An animal on basal diet alone for 20 days. The odontoblasts seem to have disappeared.

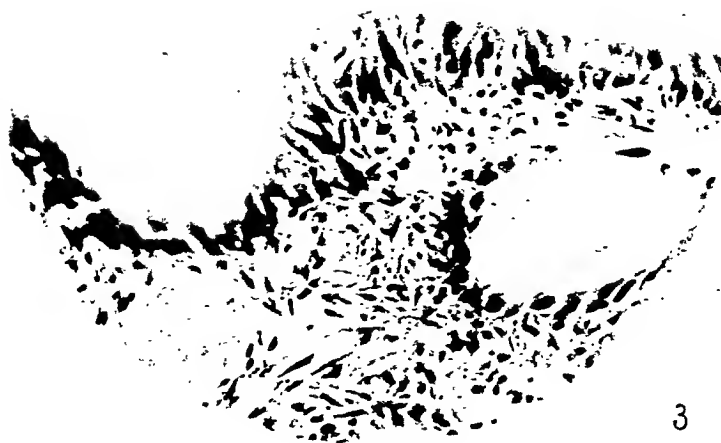
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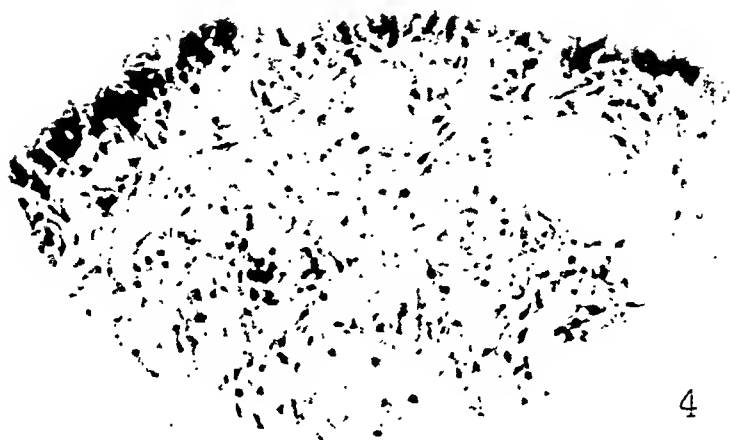
(Dalldorf and Zall: Tooth growth in experimental scurvy)

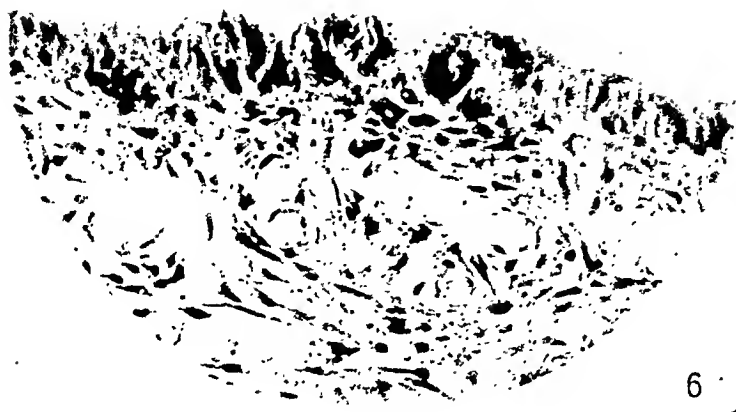


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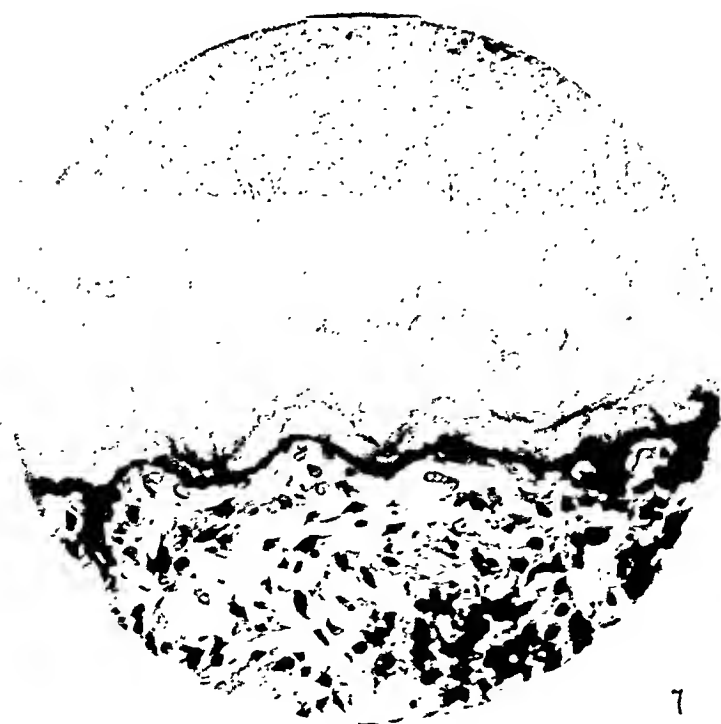


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STUDIES ON THE BLOOD VESSELS IN THE MEMBRANES OF CHICK EMBRYOS

PART I. ABSENCE OF NERVES IN THE VASCULAR MEMBRANE

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PLATE 5

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Experiments on the behavior of the blood vessels have with few exceptions been confined until now to tissue in which there are nerves. In all the organs of the body in adult human beings or in animals that have attained full growth, in which such investigations have taken place, the presence of vasomotor nerves has been demonstrated either anatomically or physiologically (1).

It is to be expected that in investigations of the reaction of blood vessels in tissues or an organ which is not innervated, the part usually played by the nervous factor can be ascertained. Experiments carried on in such preparations might make possible in a new way the elucidation of questions having clinical interest, as for instance, those arising in arterial hypertension (2) in which hypersensitivity of all vessels is present as well as in arteriosclerosis (3) in which the level of irritability is reduced and finally, in cases of inflammation (4, 5) in which the part played by the nervous system is still the subject of discussion.

On the Absence of Nerves in the Yolk-Sack of Chick Embryos

Concerning the occurrence of nerves in the membranes of chick embryos there exist in the literature, so far as we know, no references (6). There are, however, numerous investigations on the innervation of membranes of human beings and other mammals to which we intend to refer by way of comparison. The question whether the umbilical cord and the placenta contain nerves dates according to Schott (7) from the time of Galen. Reports of more recent investigations, to the

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literature of which Schmitt (8) has referred, and which are based in part on histological investigation carried out in tissues studied mainly by the method of impregnation with silver, and in part on pharmacological methods, contain the conclusion that the umbilical cord contains nerves of its own but only in that portion close to the body, and that its greater distal portion as well as the placenta are free. Ikeda (9) recently, at the suggestion of Aschoff, has reinvestigated this matter and has confirmed the statements of earlier authors.

We have attempted to solve the question as to whether the membranes of chick embryos contain nerves in part by physiological, in part by histological investigations.

Physiological Experiments

Technique.—The experiments were conducted in a constant temperature chamber at 38°C. Sources of error due to fluctuation of temperature are therefore avoided. Eggs in all stages of development, but mostly those 3 to 4 days old, were studied. In older eggs the separation of the membranes is more difficult but can, after practice, be conveniently managed. Eggs were opened at the air-chamber end without turning or jarring them, as much of the shell as was necessary to expose the vascular region being removed by means of forceps. The embryo surrounded by sufficient of its membranes could by this means be exposed. The loss of albumen was, of course, prevented. The manipulation of the egg on opening it must be gentle; otherwise the blood vessels will be found even at the beginning of the experiment to be contracted, so that weak stimuli would lead, while they are in this state, to erroneous inferences.

For electrical stimulation the usual form of a reliable inductorium was used. The source of current was an Edison storage battery. When drugs were injected the point of the needle was inserted laterally until it came to lie under the membranes, since preliminary experiment showed that better and more uniform absorption took place than if these substances were applied drop-wise to the surface of the preparation. The experiments were brief, never lasting longer than 10 minutes so that errors due to desiccation might be avoided. The observations were made with a Zeiss dissecting microscope furnished both with binoculars and bin-objectives. The embryos were protected from excess heat by placing a water filter between the source of light and themselves.

We observed the behavior of blood vessels of the yolk membrane in some 1600 eggs at various ages. The experiments which we report now, selected from the great number which were performed, permit our making the following statements concerning the innervation of these vessels.

1. One locus of the membrane was touched several times with a fine

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glass rod. Depending on the number of contacts and their strength, either constriction or dilatation took place. These stimuli brought about this effect both in capillaries and in small arteries. Even the strongest stimulus of this mechanical sort brought about no change in the immediate environment. The effect of the stimulus was strictly confined, wherever it was exercised, to the site which was stimulated.

2. A faradic current was employed to stimulate a given locus of the vascular layer, electrodes of varying diameters being used. Both the smallest arteries and the larger arterial branches were stimulated. One electrode consisted of cotton wool moistened with Ringer's solution which was placed on the membranes at a distance from the vascular zone, that is to say, beyond the marginal vein. The other electrode had the diameter of a hair. As the result of the stimulus, dilatation occurs either in a capillary or at one point of the artery, but is so small in extent as to be recognized only microscopically. If an electrode having a larger diameter is used the effect is apparent in a larger area. In all cases the effect of stimulation, whether it be contraction or dilatation, is to be recognized only at the point at which the stimulus is applied or, when the strongest current in our scale was used, in the immediate neighborhood. Involvement of neighboring regions or a propagation from the capillaries to larger arteries, or propagation in the reverse direction was never noticed.

The following experiment was made with the view to extending these observations and to detecting the possible existence of conduction over nerve paths:

The central end of an artery which led to a vascular area which we wished to stimulate was tied off and the whole vascular region was isolated by a circular incision so that every connection with the embryo was severed. In this isolated vascular region electrical stimuli were made just as before. The result of stimulation was the same as when the region was still in continuity with the embryo. On account of the absence of a circulation, that is to say of additional blood, the column of blood in the vessel when it was dilated, the degree of dilatation depending, of course, on the strength of the stimulus, appeared to be paler than before. Measurement of the diameter microscopically indicated, however, the same degree of dilatation as when the connection with the embryo was intact.

3. A fine hollow needle was inserted outside the vascular area and then pushed forward so that its opening came to lie beneath it.

Sodium iodide solution 6 per cent was injected in the amount of 0.1 cc. Dilatation of the vessels occurred at once but only at the point of injection. If larger amounts were injected a larger region was affected. If the same amount of saturated solution of sodium iodide is injected, stasis in the region of the drop immediately occurs. On account of the stagnation of blood which results, blood accumulates in the portion of the vessel central to this point. This appearance quite obviously represents a secondary phenomenon. The effect of the stimulus itself remains confined to the site of the stimulus. It appears to be unnecessary to report our experiments with other chemicals. The result was always the same—the stimulus affected merely the locus at which it was applied. If larger amounts were injected there was always the possibility that after absorption of the substance, and distribution in the circulation, a result would occur at points far removed from the point of stimulation and would there give the impression that a stimulus had been applied. But when small amounts are used and are confined to a small area the effect of the stimulus takes place only at the point of stimulation.

These observations differ from those of blood vessels which are known to be innervated. In the skin there occurs with every stimulus of low intensity an easily demonstrated erythema, an effect of the stimulus which can be seen far beyond the point of stimulation (Ebbecke (10), Lewis (11)). Reactions like this can be understood only as the result of an action in which the nervous pathways are involved. In the pancreas of rabbits for instance, Ricker and Regendanz (4) found that the effect of strong stimuli involved a larger region than the point stimulated in contrast to that of weaker ones, the effect of which was merely local. In such cases the result is brought about by a reflex (nervous) action. In our experiments we could never bring about an effect beyond the region of stimulation even if strong currents were used. The restriction of the reaction of the blood vessels in the vascular areas in our preparation to the site of the stimulus suggests strongly that there are no nerves in the vascular layer of these embryos.

Among the drugs that have been tested which have an effect upon the blood vessels adrenalin is the only one which has an action conspicuously different from the ordinary on the vascular area of these membranes.

Adrenalin, in a solution of 1 per mille, was injected through a needle at a point just under the vascular area since it had been learned that adrenalin applied to the surface had no effect even when large amounts were used. 0.1 cc. of a 1 per mille solution brought about no visible contraction. It was not until an amount of 0.4 cc. or more had been injected that a contraction of the blood vessels took place, but even then the effect was never immediate. Indeed the earliest evidence of action was observed only after $\frac{1}{2}$ minute. In most experiments contraction did not begin until the end of 4 to 5 minutes.

But even large doses like those just mentioned were not effectual in all cases. Of 38 observations, constriction took place in 25, in 4 the effect was uncertain, and in 9 there was no effect at all. Because of the weak action of the drug we were obliged to use relatively speaking unusually large amounts of fluid, 0.5 cc. or more. Control experiments with equally large doses with physiological salt solution of about the same hydrogen ion concentration, had as a matter of fact a very similar result. We are inclined to attribute the effect to the solvent therefore rather than to adrenalin. Even if we were to attribute the effect observed to adrenalin, the extent of it was remarkably small.

The contrast between this result and that in tissues which are normally innervated is striking. For in the latter it is easy to obtain a reaction everywhere by the local application of 0.1 cc. of a 1 per cent solution. The small effect in the vascular area of our preparations is accordingly altogether unusual.

Results similar to ours have been reported by Schmitt (8) in the case of blood vessels of the placenta which were treated by a perfusion method. If into the blood stream of the perfused placenta 1 cc. of suprarenin in the concentration of 1 to 1000 was injected, no effect was observed on the rate of flow in the vessels. Since it is generally recognized that the action of adrenalin takes place by virtue of its effect on nerves or on mechanisms of a nervous nature, the small effect of adrenalin on the vessels of the vascular membrane of chick embryos may be taken to represent further evidence that a nervous mechanism is wanting in these structures.

Anatomical Evidence

We have examined the embryonic membranes of chicks by means of a large variety of histological methods using the mesentery of rabbits as well as other organs, amongst them the skin, heart and lungs, by way of controls. Our best results were obtained by the use of Bielschowsky's method of silver impregnation in block, and also with the rongalite white method.

1. *Investigations with Bielschowsky's Method.*—We impregnated by Bielschowsky's method, 6 embryos and their membranes aged 3, 4 and 7 days, according to the recommendations of Schmorr (12), and cut them in serial sections. In the case of older embryos only single sections of the membranes were made. As early as 72 hours after the beginning of incubation, small, fine nerves were found in the region of the brain and in the ganglia of the head. At the end of 96 hours nerves could be found everywhere in the brain and spinal cord as well as in the spinal ganglia (Fig. 1). At the end of 7 days nerves were to be found in all parts of the embryo.

Contrary to the situation in the embryo itself, nerves were to be found nowhere in the membranes. In those sections in which the transition from embryo to membranes could be clearly seen it was apparent that the nerves were confined to the structure of the embryo and did not proceed over to the membranes, as for example in a specimen 7 days old (Fig. 2, b). Small single fibrils were seen stretching beyond the region of the lateral limiting sulcus (Fig 2, a) but in no section could they be traced to the membranes themselves. The last of the nerves found in Fig. 2 is shown by means of greater magnification in Fig. 3.

2. *Investigations with the Rongalite White Method.*—Whereas the silver impregnation method according to Schmorr's description gave always completely satisfactory results, difficulties were encountered at first with the rongalite white method. The only investigators who seem to have used this method have been Kreibich (13) and Glaser (14, 15). They succeeded even in cases where other methods failed in obtaining first-rate results both with intra- and supra-vital staining. Since an intra-vital technique was impossible on account of the small size of the objects, we have used the supra-vital method only. The mesenteries of rabbits were again used as controls. Since both the authors just mentioned have described the method in a cursory way only, we have decided to describe it in detail, according to the procedure which gave us the best results.

Rongalite white, which is methylene blue reduced by means of rongalite, was prepared in the following way: 1 gm. of pure methylene blue was dissolved in 200 gm. of distilled water, was heated, and then 6 gm. of rongalite and 15 drops of hydrochloric acid were added. This solution was boiled until it became clear and was then filtered. Staining was carried out in the following way. Organs were taken from the embryo immediately after death and were bathed in physiological salt solution. Rongalite white 5 per cent to 10 per cent was next added. After pieces of tissue had been stained for 45 minutes they were fixed in ammonium molybdate 5 per cent. After thorough washing they were placed in absolute alcohol and xylol and were then drawn up on to glass slides.

By this method we examined the membranes of 19 embryos of 3, 4, 8, 9, 10, 13, 18 and 20 days' incubation after they had been stained and a control provided for each membrane. Whereas in the mesentery, nerves could be beautifully demonstrated everywhere, none could be found in any of the membranes.

DISCUSSION

It appears then from our histological investigations that we were unable by means of the two best methods available to us to detect nerves in the membranes of the chick embryo. Neither did we find primitive cell processes which might be regarded as undifferentiated nerves. Our results therefore are in complete agreement with those which have been published in the case of the placenta both of man and of other mammals.

Is the conclusion to be drawn that the membranes contain no nerves? The objection may be made that since we are dealing with embryonal tissue we should not expect to find fully differentiated nerves. But the fact is that these tissues pass through a definite cycle of development, the course of which we have been able to follow from beginning to end, and that we have been unable to demonstrate nerves in any stage of their development, although the embryos themselves, from on the 3rd day on, always contained them.

Our anatomical results are in agreement with our physiological investigations. The absence of a reaction in the immediate neighborhood of an area, even when strong stimulation has been applied to the area itself, and the atypical behavior of the blood vessels in adrenalin experiments, can be understood only as resulting from the absence of nerves.

CONCLUSION

The agreement of physiological experiment with anatomical findings justifies our conclusion: the blood vessels of the vascular membrane of chick embryos do not contain nerves.

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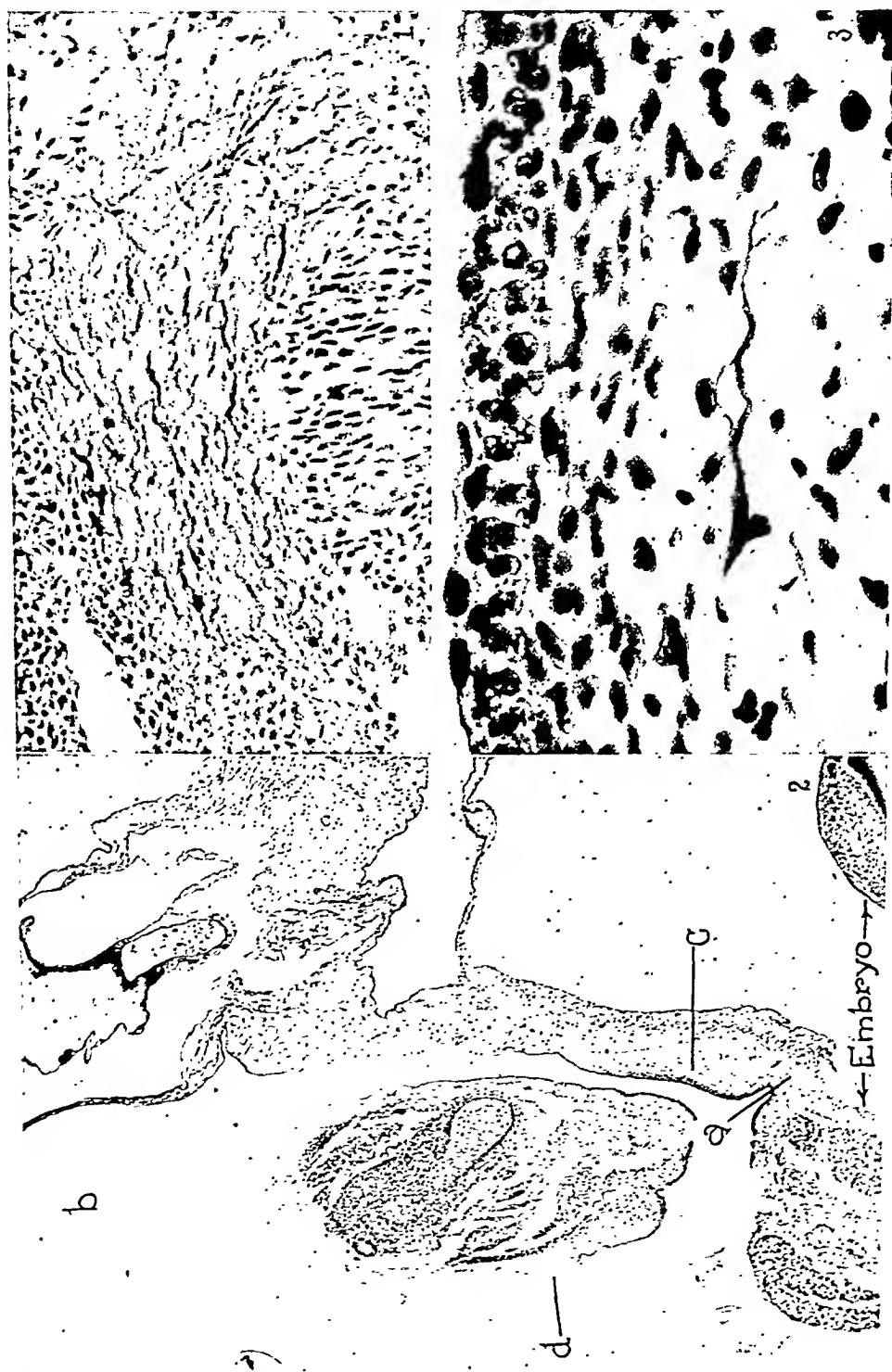
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EXPLANATION OF PLATE 5

FIG. 1. Chick embryo No. 177. Incubation period 4 days. Neurofibrils in a ganglion-anlage of the body. Silver impregnation. $\times 450$.

FIG. 2. Chick embryo No. 173. Incubation period 7 days. A cross section through the body is shown at the point of transition from the embryo to the membranes. At (a) the lateral limiting sulcus appears, and at (b) the amnion and chorion. The neurofibrils farthest away from the body (Fig. 3) were found at (c). At (d) there is a cross section of an extremity. Silver impregnation. $\times 40$.

FIG. 3. The region (c) indicated in Fig. 2 is shown. $\times 1000$. It exhibits the most distant peripheral nerve found in this section.



STUDIES ON THE BLOOD VESSELS IN THE MEMBRANES OF CHICK EMBRYOS

PART II. REACTIONS OF THE BLOOD VESSELS IN THE VASCULAR MEMBRANES

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In recent years there has been lively discussion on the behavior of the vascular system especially concerning the dependence of reactions of the vessels on the nervous system (1, 2, 3, 4). In order to advance knowledge of this matter and to make clear what actually takes place, and to attempt to indicate the significance of these processes, it has become necessary to enlarge this study by examining a new and a greater variety of material. The blood vessels of the vascular membranes of chick embryos seem especially adapted to the ends in view. Membranes containing the vessels can be easily examined both microscopically and macroscopically after removing the shell and the shell membrane. Stimuli of any desirable kind may then be easily applied. Since it has been shown (Part I) that these tissues contain no nerves, the course of the reaction takes on a special interest.

A. The Effect of Electrical Stimuli of Varying Strength on the Blood Vessels

Faradic Stimulation.—The technique used was the same as that employed in Part I. The electrode consisted of two copper wires each a millimeter thick and separated a distance of 1 mm. by means of collodion. The points of the electrodes, which alone came in contact with the tissue, were polished together with the collodion in which they were embedded, by means of sandpaper. Eggs were used after having been incubated 4 days.

In order to facilitate comparison, stimuli were made in the following experiments always at the same place, namely, the final pre-

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capillary branches of the artery which passes upwards towards the head of the embryo and to the right.

Experiment 1.—A very weak faradic current just strong enough to create a sensation of prickling on the moistened lips was used. After the electrodes had been placed, without exerting pressure, upon the appropriate vessels, current was allowed to flow through the electrodes for 10 seconds. They were then removed for 10 seconds in order to observe the effect which had been created, and then were replaced for a further 10 seconds. The duration of the stimulation was measured with a stop-watch. After the stimulus had been applied for 20 seconds, dilatation of the vessels appeared. The flow of blood in them continued to be rapid. In the surrounding vessels there was no change. We designate this stage in which there is dilatation with rapid flow "the stage of fluxion," adopting Ricker's term. 1 minute after the end of stimulation, the original diameter and rate of flow had returned.

From this experiment we made the following inference: The weakest possible faradic current brings about a brief stage of fluxion.

Experiment 2.—In this case a faradic current of medium strength which causes merely a burning sense when applied to the lips was used. The site of the stimulus was the same as in Experiment 1. The duration of the stimulus was 20 seconds, during the course of which there was an interruption of 10 seconds. At the end of 20 seconds, contraction of the blood vessels developed. The rate of flow was rapid. If the stimulus was continued for 20 seconds longer, the blood vessels shut. In the capillaries just peripheral, the flow ceased, so that the blood in them was motionless. Others were empty. The larger arteries central to these appeared unchanged. The flow of blood just proximal to the point of contraction was rhythmical. 5 minutes after the end of the stimulus the original diameter of the vessel was reestablished and the rate of flow was as before.

We made the following inference: A faradic current of medium strength brings about contraction of that arterial branch which has been stimulated.

Experiment 3.—A faradic current which was just strong enough still to be borne when applied to the lips, and which caused an unpleasant sensation of burning was used in the same way and at the same site as in Experiments 1 and 2. After 20 seconds there appeared a spindle-shaped dilatation, the degree of dilatation being greater than in Experiment 1. The rate of flow was at first slow, and then stopped after a number of seconds at a point further out than the site stimulated. Central to this point the dilated vessel was filled with blood. The blood corpuscles agglutinated so that they were no longer individually identifiable. Adopting Ricker's term we designate this "the stage of stasis." Capillaries peripheral to the site of

stimulus were empty. Just central and just peripheral to that portion of the affected vessels which was dilated, but within the region of influence of the stimulus, the arterial branch was contracted; in consequence, the dilated portion appeared as distinctly spindle-shaped. The spindle-shaped dilatation and the contraction of that part of the vessel just central to it appeared to occur synchronously. The capillaries beyond, that is to say peripheral to the vessels so affected, were empty. Transition between the dark red region of stasis and that region beyond which appeared light owing to the absence of blood could be recognized macroscopically as a sharp line. Stasis persisted throughout the observation and could not be influenced by any new stimulus.

From these findings we have drawn the inference that a strong faradic current brings about stasis.

In order to ascertain whether and to what extent dilatation depends on the pressure of the inflowing blood stream, we carried out the following experiment.

We placed a pincette which shut off the flow of blood on a small artery. A stimulus of the same strength as that employed in Experiment 3 was then applied to the artery. Dilatation of the same degree took place. The blood in the dilated vessel was, however, a lighter red since it was distributed in a greater space. After 2 minutes the pincette was removed. The dilated vessel was thereupon filled with blood and the spindle appeared to be quite full.

There appears, therefore, to have taken place, genuine primary dilatation with filling as a secondary phenomenon. The dilatation may become so great and the spindle so filled with blood that in young specimens the total volume of blood contained in the embryos has been seen to collect in the dilated area. Under these circumstances the heart continued to beat but appeared as a mere shadow, seeing that it was empty of blood.

Under similar experimental conditions a weak faradic current brings about dilatation of the arterioles of the vascular membrane with increased rate of flow, the stage of fluxion, a current of medium strength, contraction to the point of closure, and a strong current stasis of the blood in a dilated vessel. It appears therefore that the reaction depends upon the strength of the current. It is necessary next to ascertain whether other stimuli have the same consequences.

B. *The Effect of Various Other Stimuli*

(a) *Mechanical Stimuli*.—In this case the stimulus consisted of touching the locus with a blunt glass rod. The site of the stimulus was as before. Because of the greater size of the glass rod, small arteries as well as the capillaries were involved.

The *gentlest possible* contact was established 3 times in 1 second, the intervals between being naturally brief. As a result the small arteries and capillaries dilated, becoming larger than those in the neighborhood. The rate of flow in the dilated vessels was rapid. This is the stage of fluxion. *Stimuli of medium strength* were applied 3 times, at the same rate as before and to the same site but with somewhat greater pressure. The small arteries and capillaries now contracted and disappeared. The site which was stimulated appeared as a lighter spot, empty of blood. The vessels in the surrounding region were not visibly altered and exhibited a rapid flow. 5 minutes later the small arteries again filled from the side of the heart and 1 minute later still the general appearance was the same as before the application of the stimulus. *Strong stimuli* consisted in bringing the glass rod in contact with the vessels 3 times, using quick strong strokes. An enormous dilatation of the small arteries and capillaries took place at once in the region stimulated. Centrally and peripherally, waist-like contractions were observed. The capillaries which were related anatomically to the area, but which were outside the region affected by the stimulus, were empty. An hour afterwards no change had taken place, stasis still persisting.

We drew the following inference: Depending upon the strength of the stimulus, mechanical irritation brings about a stage either of fluxion or one of contraction or one of stasis.

(b) *Ammonia*.—The site of stimulation was as before. The stimulating substance was applied through the needle of a small syringe.

Experiment 1.—When 0.5 cc. of a 1 per cent solution was injected, dilatation took place at once as well as a rapid flow of blood at the site of stimulation.

Experiment 2.—10 minutes after the stage of fluxion had been brought about as in the first experiment, the injection of ammonia (0.5 cc.) was repeated. The capillaries and the smallest arteries contracted. When a third injection of the same amount was made there was moderate stasis in the capillaries whereas the small arteries were contracted.

Experiment 3.—When 0.1 cc. of a 5 per cent solution of ammonia in Ringer's solution was injected slowly, the arterial branch which was affected dilated enormously at once while complete cessation of blood flow through it took place. Dilatation was so complete that stasis did not develop as in the previous experiment but instead coagulation of the blood. Agglutinated cells floated about in a clear medium within the vessels.

We have drawn the following inference: Ammonia brings about the stage of fluxion, of contraction, or of stasis and hemorrhage, according to the concentration used and the period during which it acts. Strong concentrations cause coagulation (pseudostasis).

We have chosen the experiments described as examples of the effects of 3 qualitatively different kinds of stimuli. But we have also made experiments with mustard oil, with silver nitrate, with potassium iodide and with oil of camphor. In principle the action of all was the same. It was possible by grading the concentration and the dose, to bring about the same effects as when the faradic current, the glass rod or ammonia was employed. Since the stimuli used were able to produce an effect on blood vessels in tissue in which there were no nervous elements, we have been forced to conclude that they have their effect upon the cells themselves and that these have an inherent power to exhibit the motions which we have observed.

The action of adrenalin as reported in the first of these papers was inconstant. Atropin had no action at all even in concentrations as high as 1 per cent. Cold and heat had no distinct effect. For that reason we were unable to utilize different degrees of temperature as means of establishing grades of reaction on the part of the vessels.

In our experiments we have found then that with effective stimuli there is a systematic relation between the strength of the stimulus and the reaction of the blood vessels, in the sense described by Ricker in the case of blood vessels in which a nervous apparatus exists. A weak current as we have shown is followed by the stage of fluxion, one of medium strength by that of contraction and a strong one by stasis. The general rule of the relatedness of a particular form of reaction to a particular intensity of stimulus is exhibited also by the non-innervated blood vessels studied in these experiments and by the kinds of stimuli which were employed. Our observations therefore confirm Ricker's theory concerning the laws governing the relation between reactions and the strength of stimuli. In the case of blood vessels which are not innervated it appears that the strength of the stimuli not the nature of the stimulus constitutes the determining factor. The same forms of reaction were found whenever it was possible to grade the strength of the stimuli irrespective of their kind.

C. The Effect of a Given Stimulus upon Different Portions of the Vascular System

In the experiments so far reported, stimuli were always permitted to exert their effect upon vessels of a like diameter. We have next to report our study of the effects of the same stimulus on capillaries, small arteries and on arterial main stems. For this purpose we have employed faradic current, mechanical stimulus, or one of the chemicals already mentioned, mustard oil, silver nitrate, potassium iodide or the oil of camphor.

Experiment 1.—We stimulated a capillary area with a very weak current for 20 seconds. There resulted the stage of fluxion. When the same stimulus was applied to small arteries or the main stems of arteries, no visible reaction took place and we drew the following inference: A stimulus which brings about the stage of fluxion in capillaries has no effect upon arteries.

In *Experiment 2* a stimulus of medium strength applied for 20 seconds to a small artery caused contraction. A stimulus of the same strength applied for the same length of time to the capillaries caused stasis. If the same stimulus was applied for the same length of time to an arterial stem, no distinct effect ensued although the appearance of a slight degree of dilatation occurred occasionally. From this experiment we draw the inference that a stimulus which causes contraction in small arteries causes stasis in the capillaries.

Experiment 3.—If a strong stimulus was applied for 20 seconds to an arterial stem, stasis took place in the vessel which had undergone spindle-shaped dilatation. The same stimulus if applied to small arteries or capillaries likewise caused stasis. And we draw the inference that a stimulus which causes stasis in arterial stems does so likewise in small arteries and capillaries.

SUMMARY

A comparison of the effect of stimulation upon the arterial subdivisions shows that these differ in irritability. The capillaries are most irritable, then come the small arterial branches, and last the main stems of arteries, these being the least irritable. The non-innervated blood vessels of the vascular membranes exhibit therefore the behavior described by G. Ricker in the case of those which have a nervous apparatus. We have demonstrated accordingly similar properties in vessels which are not innervated as in those which are. Certain differences exist however. The action of adrenalin on the vessels of the vascular membrane is inconstant and certain other sub-

stances are wholly ineffective. Ricker's experiments have advanced knowledge on the regularity of the vascular reactions and have paved the way for these experiments of ours. The result of our experiments with vessels free of nerves suggests that a reexamination may be fruitful of the mechanism which obtains in innervated vessels. There can be no doubt that nerves play a rôle in the behavior of vessels; it appears now to be necessary to define more accurately precisely what this is.

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STUDIES ON THE BLOOD VESSELS IN THE MEMBRANES OF CHICK EMBRYOS

PART III. ANATOMY AND PHYSIOLOGY OF THE BLOOD VESSELS AT DIFFERENT AGES*

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The development of the vascular system of the membranes of chick embryos passes through a definite cycle. It can be followed from its inception in the blood islands, through the stages in which the blood vessels are finally developed, to the time when it is discarded at the time of hatching. It ought to be possible therefore to correlate changes in function with the anatomical changes. We hoped to obtain an insight into the alterations which take place in both, and to do this the more readily owing to the short duration of the whole cycle.

Physiological Experiments

The irritability of the vessels was studied by applying stimuli of the same strength to them from their inception to the time of hatching. The experiments were carried out in eggs that had been opened as in Parts I and II of these studies.

It was necessary to introduce a slight modification in technique in the case of older embryos for in them it is more difficult to remove the membrane than in embryos 5 days old. The eggs were therefore kept on end, the air-chamber upwards, for 12 hours before the beginning of the experiment. With practise it became easy after opening the shell in the region of the air-chamber, to remove the shell membrane with fine forceps and scissors. The blood vessels then are as easy to approach as they are in younger embryos in which they are disposed along

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the long axis of the egg. The stimuli employed were a faradic current of known strength, sodium iodide, ammonia and mechanical stimulation with the use of a glass rod. As in our earlier experiments we ascertained which strength of stimulus it was necessary to employ in order to secure stasis in an egg after 3 days of incubation. We then chose a stimulus based on this, but weaker, maintaining all the stimuli which we employed at the same strength for all other ages. On account of the similarity of our observations it is necessary to describe only our results.

At the time when there are only blood islands and no regular flow of blood, contraction and dilatation do not occur. At this early age the occurrence of coagulation as the result of the use of chemicals in strong concentrations can naturally not be observed. Our observations begin therefore with the behavior of blood vessels at the time when a regular rate of flow begins, namely, on the 3rd day. All stimuli which bring about stasis in arteries of the membrane on the 3rd day of incubation produce the same dilatation in the same length of time in arteries of the same size and in the same way in all eggs of all later ages. The criterion of a strong stimulus has been described in previous sections of these papers and may be defined as follows: In the case of a faradic current it is one which on moist lips causes an unpleasant sensation of burning. In the case of sodium iodide a concentration of 6 per cent in the amount of 0.1 cc. and in the case of ammonia a 5 per cent solution of the same amount. We chose a weaker current, as has been said, one which in small arteries causes contraction and studied the reaction to it through all ages up to the 19th day, that is to say, shortly before hatching of the egg. In all stages of development the effect of stimulation of arteries of the same caliber is the same, namely, contraction.

A difference in the manner of reaction is always visible. Capillaries react to weaker stimuli than do arteries. In this sense specimens which are younger differ from older ones. In specimens 3 and 4 days old, all the vessels respond to stimuli which in older embryos affect only those vessels which have the same small dimensions as the young ones. The larger vessels in older embryos require stronger or longer stimuli. For example, stasis was observed in all arteries in 3 to 4 day old embryos as the result of applying an electrical stimulus of a certain strength for 20 seconds. In specimens 18 days old on the contrary this stimulus applied to large arteries required twice the dura-

tion, namely, 40 seconds or a correspondingly stronger stimulus for 20 seconds to bring about the same effect. This striking difference is to be explained in terms of histology.

Anatomical Investigations

There are satisfactory investigations by Popoff (1) and H. Virchow (2) on the distribution and branching of blood vessels of the vascular membrane of chick embryos at various ages, but a comparative histological study of these vessels at all stages of development has not so far been published. In especial nothing is known on the presence of elastic tissue nor of the appearance of degeneration in the older aged groups. In the very early stages of development studies have been made by Sabin (3) both in living preparations and histologically. According to her investigations a continuous differentiation of mesenchymal cells to angioblasts takes place in the first 2 days. These cells develop first in the membranes which are of interest to us. Angioblasts differentiate further during the course of the 3rd and 4th days of incubation. The angioblasts give rise to endothelium, blood plasma and blood islands. Only those structures are called blood islands which develop hemoglobin and become erythroblasts. The lumina of the blood vessels come into being through a process of cytolysis either in the centers of the cell masses or in the cytoplasm of single cells.

Our histological description begins at the 3rd day of incubation when the flow of blood in the vascular membrane has become established.

Technique.—Freshly opened eggs were fixed in formalin, Mueller formol or Zenker's solution. Blocks of paraffin or of gelatin were then made which were cut in series from the marginal veins to the embryo. Specimens of various ages were so prepared, cut either horizontally or transversely. They were stained with hematoxylin and Van Gieson's solution, resorcin-fuchsin and Sudan hemalum.

Description of Embryos 4 Days Old.—Radiating from the embryo there are ridges and folds which press into the yolk. In these folds the blood vessels run. The arteries lie in the folds nearer the yolk. They consist of single rows of flat or spindle cells, the circumference of the wall being the length of 10 to 12 cells. In the surrounding area there is a loose mesh network in which there are large nuclei. These cells do not belong to the arteries since they are also to be found without relation to them. Within the vessels there are free nucleated cells either round or spindle-shaped depending upon the aspect which they present. Laterally from

the arteries lie two veins. In comparison with the arteries they have a characteristic form. They are polygonal or flattened and adapt their shape to the space which is available, in this case, half surrounding an artery. Their wall is thin and consists of a single layer of cells. Arteries and veins are immediately juxtaposed so that at such places two layers of flat cells are to be found. There are no elastic fibers and no collagen. All vessels that belong to the yolk have the same form and are to be distinguished only by their size.

Description of Embryos 10 Days Old. Vessels at a Distance from the Embryo.—In the interval the dimensions of the arteries have increased to a five-fold extent. The wall turned towards the yolk consists of a single layer of cells. They contain a light staining protoplasm. The nucleus stains darker and contains many dark points arranged about a larger, more compact, area within. The relation of arteries and veins is less intimate; the veins run, at least in part, an isolated course.

Vessels near the Embryo.—Arteries have come to differ from veins through the distinct difference in thickness of their walls. On cross section the arteries appear to be round in contrast to the veins which are capable of taking on a variety of forms. The veins surround the arteries to the extent of a half to three-quarters of their circumference. The artery is embedded in the loose web-like tissue of the embryonic membranes. Their walls are about 5 cells thick. Their arrangement is lamellar. The nuclei are arranged in the long axis. The protoplasm appears more concentrated and is less in amount. The inner layer consists of somewhat taller endothelium. Between the cells delicate fibers stained bright red with Van Gieson's solution are to be found. There are no elastic fibers and no fat. The veins exhibit, surrounding the single row of cells of which they are formed, a layer of cells somewhat loosely meshed, the cells being large and composed of light staining protoplasm. Between the largest and the smallest arteries transitions occur ranging in dimension from arteries presenting two to three layers of cells to those of the largest size. The arrangement is always circular and never longitudinal. The smallest blood vessels, namely capillaries, often appear close to arteries, touching their outermost layers, but do not penetrate their walls.

Description of Embryos 14 Days Old. Vessels at a Distance from the Embryo.—The smallest arteries are built like capillaries and are to be distinguished from them only according to their size. They are of exactly the same form and structure as were those described in embryos of an earlier age. Nearer the embryo there are likewise to be found larger arteries of 2 to 5 layers.

Vessels near the Embryo.—A large artery of about 12 layers is to be found, the lumen being lined with a single layer of endothelium. Surrounding this is a longitudinal layer three cells thick. Beyond is a circular layer seven cells thick. Outside the vessel there lie connective tissue cells irregularly distributed and loose meshed, the cells containing small nuclei. The protoplasm of the cells in the arterial wall stains brown with hematoxylin and Van Gieson. These are young muscle cells. Distributed among them there are a few collagen fibers stained pale red. In the loose meshed tissue which is to be regarded as the adventitia there are

a few collagen fibers but there are no elastic fibers and there is no fat. The veins have a larger lumen than the arteries and are built of single layers of endothelium and about two layers of cells. The cell layers appear to be looser meshed than in arteries.

Embryos 18 Days Old. Description of the Vessels Far Removed from the Embryo.—Arteries built like capillaries are to be found as well as small arteries such as have already been described.

Vessels near the Embryo.—The arterial trunk described in embryos 14 days old is to be found also here. The wall has become thicker. The nuclei are well stained and their structure unchanged. There is no evidence of nuclear degeneration, decrease in the size of the nuclei nor indeed of any other change. There is no fat in the wall nor any elastic fibers.

At the Site of Transition to the Embryo.—The form of the artery is unchanged. There appear to be more collagen fibers. Sections stained with resorcin-fuchsin exhibit stoutly developed elastic fibers. In the outer third of the muscle layer there lies a broad band of elastic fibers passing in part into the adventitia. This zone consists of about five layers of short twisted elastic fibers which form a mesh-work. There is a second layer of elastic fibers very fine in structure, which forms the internal elastic layer close under the single layer of endothelial cells. This structure also is not closely organized, but consists of single short slightly curved fibers which run circularly. Neither fat nor any other form of degenerative substance is to be found. This part of the artery exhibiting elastic fibers should no doubt be reckoned as being part of the embryo. This is the portion in which, as has been described in Part I, nerve fibers were found.

In the embryo itself in the early stages there are definite elastic rings in arteries of very much smaller dimensions than in the vascular membrane. The proof that elastic fibers occur in arteries within the embryo, whereas in those without there are none, is certain since in one and the same section both the embryo itself and the vascular membrane are frequently found.

SUMMARY AND DISCUSSION

On the basis of the anatomical studies presented the following inferences or conclusions are drawn.

1. In the course of development there appear in the vascular membranes of chick embryos arterial vessels of all calibers, namely, capillaries, small arteries with 2 or 3 cell layers, and large arteries formed of endothelium, longitudinal and circular layers of muscle and adventitia.

2. In none of the stages are elastic fibers developed. Only in the most central portion of the umbilical artery, in that portion namely

which is to be regarded as belonging to the embryo, are elastic fibers discoverable.

3. The structure of capillaries is histologically the same at all stages. The small arteries of embryos 10 days old resemble histologically those of 18. At no stage of development are appearances of degeneration nor of fat to be found in arteries.

When the physiological results of our investigations are compared with the anatomical ones the following comments may be made. In respect to Paragraph 1 of the anatomical results we may remark that when we study the different forms of the wall of arterial vessels the most delicate vessels consisting of single cells exhibit the greatest irritability. Those which are built of 3 to 4 muscle layers are less irritable. Stouter vessels appearing for the first time at 10 days of incubation require stronger stimuli to bring about the same reaction. In respect to Paragraph 2 of the anatomical results we may make this comment. The absence of elastic fibers in all arteries of the embryonic membranes throughout the period of their development is important in defining a physiological property of the larger vessels.

The medium and larger vessels, beginning with the 4th day of incubation, contract differently from normal adult human arteries. In the contracted state they appear in cross section not as small replicas of larger circular structures, but take on a new form. During the course of contraction they become flat and appear band-like as would a garden hose when it is compressed by a weight. In examining a vessel so contracted one sees on rotating the vessel either a broad side or a narrow one. It is for this reason that such arteries appear alternately narrow as a line or broad as a band. It is not until the narrow artery is elevated with a hook that its uniform band-like nature becomes evident. The absence of elastic tissue, the presence of which in all probability is mainly responsible for the usual shape of arteries on cross section, permits one to see how the phenomenon which has been described may come about. Concerning Paragraph 3 of the anatomical conclusions we have this to say. According to the histological investigation a stage of degeneration is wanting in the blood vessels of the embryonic membrane in a sense in which one is accustomed to see such changes in other blood vessel systems during the course of life. On the day of hatching the constituent cells and fibers

of the arteries of all calibers are anatomically the same as in their early development. These vessels do not die as the result of aging: The nutrient fluid ceases to flow because of contraction of the umbilical vessels. The blood vessels die in complete possession of their physiological irritability and anatomical integrity. The unaltered irritability of blood vessels of the same caliber at all ages is consonant with their unaltered anatomical structure.

We wish to express our thanks to Miss Nelda Ricci for technical assistance.

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STUDIES ON THE BLOOD VESSELS IN THE MEMBRANES OF CHICK EMBRYOS

PART IV. MODIFICATION OF IRRITABILITY OF THE BLOOD VESSELS

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We studied the reactions of the blood vessels in the vascular membranes of chick embryos in Part II of this series. There we described changes which took place quantitatively and qualitatively in response to stimuli. It was our care in the earlier experiments to maintain the environment constant. In the present experiments we intended conversely to keep the stimulus constant but to change the environmental conditions. The question we have asked ourselves is, of course, whether with the same stimulus a difference in the irritability of the blood vessels can be brought about by altering the environment.

To obviate the danger of drying, due to the increased length of time required by these experiments, a substitute procedure was devised. We placed the eggs in Ringer's solution through which oxygen was bubbled. It was, of course, necessary first to be certain that this arrangement did not interfere with the viability of the embryos.

Experiment 1.—Eggs were opened on the second day of incubation, the albumen for the most part was drained off, and the yolk was laid in the Ringer's solution through which, as has been said, oxygen was continuously bubbled. At the beginning of the experiment the embryo with its membranes was 11 mm. in diameter, the blood vessels were not yet developed, and the embryo itself white in color. 24 hours after the beginning of the experiment the embryo and its membranes measured 14 mm., the heart was red and contracted 120 times per minute. In the blood vessels there was flow of red blood. At the end of 48 hours the embryo and its membranes measured 16 mm., the heart beat at the rate of 120, and there was

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vigorous flow in the vessels. On the 3rd day, the rhythm of contraction of the heart was irregular.

It is clear from these observations that the embryo including the circulatory apparatus grows normally for 2 days in this solution.

The demonstration of the viability of embryos and their membranes was sufficient to warrant the performance of experiments, the duration of which in no case exceeded 1 hour. We needed, of course, to be certain that oxygen in the concentration in which it was used exercised no effect on the irritability of the blood vessels of the embryonic membranes.

Experiment 2.—This was of a preliminary nature and was repeated in each case before proceeding to further tests. After opening the egg a branch of an artery was stimulated. Stasis occurred after the stimulus had been applied 3 times for 10 seconds. Then the preparation was irrigated with Ringer's solution through which oxygen was bubbled for 5 minutes. The vessel was again stimulated and stasis occurred again after 3 successive periods of stimulation each 10 seconds long.

We concluded that Ringer's solution through which oxygen was passed did not alter the irritability of the blood vessels.

We used carbon dioxide of known concentration in our experiments. It appears from the experiments of Hammett and Zoll (1) that saturated solutions of carbon dioxide bring about constriction of the vessels. Contraction takes place if the solution saturated with gas is injected under the membranes, as in the experiments of Hammett and Zoll, as well as when the surface is bathed with Ringer's solution which during this process is exposed to carbon dioxide in the usual fashion by bubbling the gas through the solution. These results we were able to confirm. We reduced, therefore, the concentration of carbon dioxide in Ringer's solution to a point at which it had no longer an effect upon the blood vessels.

Experiment 3.—Ringer's solution was placed in a glass burette having a capacity of 50 cc. Through this, air containing 5 per cent of carbon dioxide was bubbled so that 60 bubbles passed through in a minute. The egg after it had been opened was bathed with this solution. At the end of an hour the contractions of the heart remained unchanged, the rate being 128 per minute. No change took place in the diameter of the vessels, nor in the flow of blood through them.

We concluded that bathing the membranes with Ringer's solution containing carbon dioxide, the hydrogen ion concentration being between 7 and 6, would occasion no change in the rate of the heart or in the diameter of the vessels.

In view of the preliminary experiments which have just been described we adopted the following plan of operation.

A burette was provided with: (1) a tube through which oxygen was introduced, (2) a similar one for the delivery of carbon dioxide, and (3) a funnel through which salt solutions could be delivered. A tube led from the burette to a small glass cannula from which the solution designed to bathe the egg was permitted to drop. The perfusion stream was directed so that it fell not upon the embryo itself nor upon its membranes but on the surface at a distance from them, yet in such a way that the stream passed over the vascular layer. Direct impact of the fluid would have occasioned a mild stimulus the effect of which would have been characterized as we now know by dilatation of the vessels and a rapid rate of flow, the "state of fluxion."

Stimulation was supplied by means of a faradic current, the source being an Edison storage battery. To be certain that the current was constant, its voltage and amperage were continuously observed. The electrodes consisted of two copper wires a millimeter in diameter, placed a millimeter apart. With the exception of their poles they were carefully insulated. Observations were made with a binocular binobjective Zeiss microscope.

We measured the duration from the beginning of stimulation to the time of occurrence of stasis. To recognize this stage presented no difficulty because of the magnitude of the effect. Nor did this observation lead to error for the effect did not disappear and itself, therefore, served to control the observation. The experiments were all undertaken in a constant temperature room. Since the solutions and the gases were also kept here, the possibility of error due to alterations in temperature was excluded.

These arrangements permitted observations in which the vessels were stimulated alternately in milieux containing carbon dioxide and in others free of this gas.

After opening the egg: (1) Stimulation with a faradic current for 20 seconds was undertaken. The result was *stasis* of the blood stream. (2) When irrigation with Ringer's solution, *free* of carbon dioxide had been carried on for 2 minutes, stimulation with the faradic current was again applied for 20 seconds. *Stasis* again occurred. (3) When irrigation with Ringer's solution containing *carbon dioxide* had gone on for 3 minutes followed by stimulation with the faradic current lasting

150 seconds, *no stasis* appeared. (4) Next we irrigated the membrane with Ringer's solution which contained *oxygen* for 5 minutes and stimulated the vessels for 30 seconds. *Stasis* again appeared. (5) We irrigated again for 3 minutes with Ringer's solution containing carbon dioxide. This was followed by stimulation with a faradic current for 200 seconds, but *no stasis* occurred. Finally, we irrigated the preparation with Ringer's solution containing oxygen for 5 minutes and now stimulation with faradic current for 30 seconds only, was followed by the occurrence of *stasis*. Forty experiments like this were performed.

DISCUSSION AND SUMMARY

When faradic stimulation was undertaken of vessels irrigated with Ringer's solution, which alternately contained and was free from carbon dioxide, it was observed that the reaction was far less when the solution contained carbon dioxide. A reversal of the effect could be obtained many times. It appears, therefore, that when Ringer's solution contained carbon dioxide in the concentration described, the irritability of the vessels to electrical stimuli decreased, although carbon dioxide by itself and in the absence of the application of the stimuli, appeared to be void of effect upon the vessels. The rare, divergent results were traced to technical errors.

We attempted to discover whether the observed decrease in irritability of the vessels might not be due to the absence of oxygen. For this purpose we irrigated the vessels with Ringer's solution alternately containing nitrogen and oxygen. When nitrogen caused any change this was due to an influence on the rate of the heart and not on the irritability or reactivity of the arteries. In whatever way we tried we were unable to bring about a change in reactivity of the arteries by creating a condition of oxygen lack independently of a change in the rate of the heart beat.

We attempted to study also the effect of other acids beside carbon dioxide on the changed reactivity of the arteries. Irrigation with various concentrations of lactic acid was without result. We also employed solutions buffered with potassium and sodium phosphate. When the irrigation was undertaken with these solutions having a pH range varying from 7.7 to 5.9 we observed neither a direct action nor

one which modified the preparation in such a way as to change its susceptibility to faradic stimulation.

Important investigations have been published recently by Atzler and Lehmann (2) on the direct influence of the hydrogen ion concentration on the behavior of blood vessels. Hammett and Zoll believed that, as the result of their experiments in which they attempted to bring about stimulation with solutions of concentrated carbon dioxide, they were able to exclude the possibility of action due to acid alone and therefore ascribed to carbon dioxide a specific effect. In our own experiments the method of irrigation does not permit an inference whether, or how far, an acid effect plays a rôle in the carbon dioxide experiments. For beside the question of hydrogen ion concentration and of buffering, the question of the penetration of substances from the surface to the contractile elements of the wall of the vessels requires to be considered. Carbon dioxide has an ability, beyond that of all other substances, to penetrate through tissues (3). It may be owing to this property that we could influence the reactivity of the blood vessels with it and it alone.

This possibility must be further investigated. In these experiments, however, it was our object to show only that it was possible to influence the irritability of blood vessels experimentally. The conclusion is justified by our experiments that carbon dioxide in small concentrations reduces the threshold of irritability for electrical stimuli of the blood vessels of the embryonic membrane.

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ENHANCED PASSIVE IMMUNITY TO STREPTOCOCCUS INFECTION IN RABBITS

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We have been engaged for a number of years in studying the local intrapleural infection in rabbits produced by a strain of *Streptococcus haemolyticus* of increased and extreme virulence. These studies have shown that the rabbit dies uniformly of empyema on receiving a dilution of a 24 hour broth culture that contains not more than ten chains of the streptococcus. The resistance may be markedly increased, so that animals recover without lesions, by producing, with indifferent substances, cell accumulations in the wall of the pleural cavity provided that these accumulations be allowed to pass into the stage of mononuclear (clasmatocyte) cell infiltration (Gay, Clark and Linton).

Rabbits with a granulating pleura of this type are able to withstand many multiples of the lethal dose. It appears that this accumulation of clasmatocytes is effective not as a mechanical obstruction to infection but in virtue of the phagocytic value of its accumulated cells since inoculation in the opposite pleura, where no cell accumulation has taken place, also results in protection and can be shown to be due to a transpleural mobilization of the cells accumulated in the other cavity (Gay, Linton and Clark). It may further be shown that clasmatocyte mobilization in the peritoneum may be attracted through the diaphragm into an infected pleura and protect (Linton), and further that a transferred granulating omentum from one animal of the same species or even from an animal of another species (guinea pig into rabbit) may in a certain percentage of cases produce the same protection in a second animal. Throughout our experiments accumulations of polymorphonuclear cells have failed to produce the same protection. We feel justified in referring to this condition with the streptococcus as an "enhanced natural resistance" due to mobilization of mononuclear cells.

Unfortunately mononuclear mobilization although extremely effective in the streptococcus infection we have studied produces little or no result with some other microorganisms, for example with the pneumococcus and with *Pasturella leptisepticus*. To consider more attentively the case of pneumococcus local infection it appears (Clark) that the mononuclear cells are by no means without function, for if the pneumococci have previously been treated with an immune serum (sensitized), mononuclear accumulations suffice, and alone suffice, to protect the animal. Such sensitized bacteria are uniformly fatal for normal rabbits or for rabbits with polymorphonuclear accumulations. These observations led us (Gay and Clark, 1929) to suggest an explanation for the failure of the majority of antibacterial serums to produce striking therapeutic results. This hypothesis is to the effect that such serums, directed against the bodies of bacteria rather than their toxins, are ineffective not because they lack appropriate or sufficient antagonistic properties (antibodies) but because the cells which are readily mobilized in the actively immune animal are lacking in the animal to which the serum has been passively transferred. The experimental results herewith reported are the first contribution which confirms this hypothesis.

A series of experiments with pneumococcus infection by various routes in rabbits seemed to indicate that a combination of homologous antiserum plus normal mononuclear cells is more effective in protecting a normal animal against infection than the serum alone. However, the antiserum obtained in rabbits is in itself so markedly protective that we do not regard our experiments as convincing as yet. On the other hand a return to our experiments with streptococcus seems to show in no uncertain way that cells increase markedly the protective effect of an homologous antiserum. As long ago as 1920 Gay and Stone showed that the serum of rabbits hyperimmunized against the streptococcus and containing various antibodies in considerable concentration was relatively ineffective in protecting rabbits against intrapleural infections with the same organism even when injected simultaneously with the infecting organism. With a dosage of 1 to 2 cc. of serum only 5 out of 20 animals (25 per cent) recovered. It should be repeated that the control animals always died uniformly from much smaller doses of organisms and in all attempted therapeutic

experiments of this sort at least 200 lethal doses are employed. In repeating these experiments recently we have obtained similar results. The intrapleural administration of from 0.2 to 2 cc. of rabbit anti-streptococcus serum failed to protect 3 animals when given simultaneously and protected only 2 out of 10 animals when given 2 hours before the infecting dose. When the serum was administered intravenously from 1 to 24 hours before the streptococcus and in a dose of from 1 to 5 cc. only 1 animal out of 11 (9 per cent) survived.

From previous experiments (Gay and Clark, 1925) we find that although the serum in a single dose or several doses, beginning at the time of infection, is ineffective, protection may be regularly effected by giving antiserum 24 hours before the infection and again simultaneously with it. Under these conditions the pleural cavity becomes sterile within a few hours. On further analysis it was found that normal rabbit serum would also eventually result in sterilization although at a slower rate. We believe that we were able to show conclusively that this protective effect was associated with stimulation produced by the first dose, and mobilization produced by the second dose, of clasmotocytes in these protected animals. We did not at that time, however, point out the relation of antibodies to the more rapid result effected by the immune serum over the normal serum.

It remains to show that the presence of antibodies increases the considerably effective protection assured by clasmotocytes alone. This we have done in the following manner.

A. A series of 12 rabbits in several separate experiments were inoculated in the pleural cavity with streptococci corresponding in numbers to from 20 to 10,000 M.L.D. but previously exposed to sensitization by rabbit anti-streptococcus serum in a dosage of from 0.2 to 0.5 of antiserum to 2 cc. of a 24 hour culture. These animals all died. In other words sensitization of the streptococcus by an immune serum does not protect the animal.

B. As we have already repeated, animals with pleural cavities prepared by injection of an aleuronat starch mixture 3 days previously are usually protected against direct inoculation of at least 200 M.L.D. of the streptococcus. When the infecting dose is given in the other cavity, unaffected directly by previous inoculation, protection may be evident although the results are uncertain and not effective against so

many lethal doses as in the originally prepared cavity. In this series 7 animals were prepared in the right cavity with aleuronat and starch and 3 days later injected in the left cavity with from 300 to 10,000 M.L.D. Of these animals 4 died and 3 survived (43 per cent). Of the survivors 2 received 300 and the other 1000 M.L.D.

C. Eight animals were prepared in the right cavity as in Series "B" and injected in the left cavity with 500 to 10,000 M.L.D. of sensitized culture. Of these animals 5 survived and 3 died (62.5 per cent). Those that died had received only 1000 M.L.D., whereas 3 of the survivors had received 10,000 M.L.D.

There seems no reason to question from these experiments that a combination of clasmatoocytes (in themselves distinctly protective) and of antiserum (only slightly protective) is superior to either factor alone.

It is evident, however, that experiments of this type with sensitized bacteria do not correspond to conditions of natural infection with normal virulent organisms in spite of the fact that the sensitized cultures seem as fatal as untreated cultures.

We have previously shown (Gay and Clark, 1926) that the mononuclear exudate of an animal that has received aleuronat and starch 3 days previously and is itself protected has in the test tube no greater destructive property than the exudate (polymorphonuclear) of an animal prepared the day before and which is unprotected. In both instances the whole exudate fails to destroy streptococci but the supernatant fluid if immediately removed does have definite bactericidal properties. We further found that the mononuclear exudate of a non-specifically protected animal has no power of transferring this protection to another animal. As much as 21 cc., in divided doses, beginning simultaneously with the infection, have been given to a rabbit without result. When, however, we employ the exudate of an actively immunized rabbit we find that the majority of animals treated with even small doses ranging from 0.2 to 4 cc. are protected in a majority of instances; 15 out of 20 rabbits (75 per cent) treated in this way with immune exudate survived. The exudate may be given either 2 hours before or simultaneously with the infecting dose and apparently with equally good effect.

Supernatant fluid of such an immune exudate alone, without the

cells of the exudate, rarely protects. There were 2 survivors out of 15 animals ($13\frac{1}{3}$ per cent). It should be noted that in both of these experimental groups above cited protection seemed equally good when a 24 hour immune exudate was used as when a 3 day immune exudate (mononuclear) was employed. This seems a distinct difference from the contrast in protection in normal animals when these two grades of irritation have been produced in the pleura.

Further analysis shows that the cells alone of either mononuclear or polymorphonuclear exudate irrespective of whether they are from immune or normal animals do not protect and apparently the high degree of protection afforded by the whole immune exudate is due to the presence of antibodies in the supernatant fluid as well as cells. Such antibodies are always present though in slightly weaker titer than in the antiserum. It is evident that both cells and antibodies are necessary for if we add to the sediment of 1 cc. of either normal or immune exudate which may in turn be either polymorphonuclear or mononuclear, an antiserum or an immune supernatant fluid the majority of animals are protected, 8 out of 9 or 89 per cent. Normal supernatant fluid plus cell sediment does not protect.

For some reason when *whole* exudate either immune or normal was added to antiserum the protection was less marked, that is only 2 out of 9 animals survived (22 per cent). We are not prepared to explain this discrepancy except to say that the total amount injected in these animals was usually larger than when the sediment of cells was employed and that failure may have been due to the volume or to excess of antibodies.

The experiments so far seem to show that a highly effective mononuclear cell mobilization is still further increased in protective efficiency by the presence of an antiserum which in itself is relatively ineffective, or conversely that the action of antistreptococcus serum in the experimental pleurisy in rabbits is definitely intensified in its effect if suitable cell accumulations are present at the seat of infection. Having established this main point it remains to seek a more effective adaptation to natural conditions of infection, particularly in the line of curative serum therapy. We can only suggest certain lines of investigation on which we are at present embarked.

If a normal exudate from the rabbit is given in the pleura 18 hours

previously and an antiserum given intravenously at the time of infection the animals survive (only three have been tested in this fashion). All the controls with the pleural treatment or with pleural treatment plus normal serum intravenously at the time of infection, died.

TABLE I

Schematic Comparison of Degree of Protection against Streptococcus Pleurisy in the Rabbit Afforded by Different Methods

Preparation of animals	Dosage strept.	No. tested	Mortality	Degree of protection
Normal.....	1 M.L.D. (10 chains)+	66+	100%	0
Normal with 24° polymorphonuclear exudate.....	200+	8	100%	0
Normal.....	20-10,000 sensitized	12	100%	0
Normal with transferred normal exudate.....	200+	8	100%	0
Antiserum locally.....	1000 + M.L.D.	33	77%	±
Immune supernatant locally.....	1000 + M.L.D.	15	87%	±
Antiserum intravenously.....	1000 + M.L.D.	11	91%	±
Prepared 3 day (clasmatocyte), tested transpleurally.....	300-10,000 M.L.D.	12	50%	+
Prepared 3 day transpleural.....	500-10,000 M.L.D. sensitized	8	38%	+
Local normal serum—24°—normal serum.....	200 + M.L.D.	7	29%	++
Local antiserum—24°—antiserum.....	200 + M.L.D.	15	26%	++
Local immune exudate.....	1000 + M.L.D.	20	25%	++
Prepared 3 days, same side.....	200 + M.L.D.	14	14%	+++
Local cells (normal or immune) + antiserum.....	1000 + M.L.D.	9	11%	+++
(Local normal exudate + antiserum intravenously.....	1000 + M.L.D.	3	0	++++?)

It is as yet uncertain whether the cells of normal or immune exudate are themselves active in increasing the protection by antiserum or whether they simply stimulate the local tissue to a rapid clasmatocyte response. The latter possibility is suggested by the fact that a polymorphonuclear exudate is as effective as a clasmatocyte exudate and

yet the donor of the early exudate is unprotected. We have not yet fully determined whether dried cells are as effective as fresh cells or whether local irritation with some other protein such as aleuronat or milk may do as well as cells. All attempts hitherto to produce a general stimulation of leucocytes by intravenous injection of vaccines, both specific and non-specific, or by indifferent proteins have failed to increase the effect of antiserum on the local infection.

In short, all that we feel justified in saying at the present time is that there appears from these experiments in local streptococcus infections in the rabbit definite evidence of the possibility of an enhanced passive immunity as previously defined, produced by a combined action of an antibacterial serum, which is in itself relatively ineffective, and normal cellular exudate locally, which in itself has no effect.

Table I is designed to give a schematic indication of the various methods of preventing experimental pleurisy which we have investigated.

SUMMARY AND CONCLUSIONS

The experimental work herein reported tends to justify our hypothesis recently expressed, that the common failure of antibacterial serums to combat active infections when passively transferred to a normal animal, is due not so much to a lack of suitable or sufficient antibodies as to absence of cell preparation or mobilization in the recipient. In the case of experimental streptococcus empyema in the rabbit the course of the ordinarily fatal infection is in no wise affected by the transfer of the pleural fluid containing large numbers of mononuclear cells derived from an animal that is itself protected as a result of a non-specific irritation. The serum of a rabbit highly immunized against the streptococcus and containing antibodies for it, produces relatively slight effect in prevention or cure.

In contrast to this the pleural exudate, either acute (polymorphonuclear) or subacute (mononuclear), produced in an actively immunized animal does protect passively to a considerable degree. In a similar fashion normal exudate cells of either type in combination with the relatively ineffective antiserum give a high degree of protection.

It remains for further analysis to determine whether this form of passive immunity by antiserum enhanced by the addition of cells

depends on the vital properties of the cells transferred or on their stimulation to cell mobilization in the recipient. And furthermore the extent to which this enhanced passive immunity may be effective in cure, and whether the cure is applicable to local or to both local and generalized infection remains to be seen.

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THE BLOOD CHEMISTRY OF AN ACUTE TRYPANOSOME INFECTION

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The recognition of the fact that the blood chemistry is altered has given a new direction to the study of experimental trypanosome infections.

Most of this work has dealt with the effect of the disease on the blood sugar, owing in part to the relative simplicity with which this factor may be studied, and in part to the claim of Schern, one of the first students of these changes, that the trypanosomiasis are diseases in which the host's sugar is utilized by the parasites in a manner entirely analogous with that of bacteria growing in a test tube. The literature dealing with this point has been reviewed by Regendanz (1). Schern (2), von Fenyvessy (3), and Scheff (4), have published figures which show that the blood sugar concentration falls as the number of parasites increases, while other workers, amongst whom Regendanz (1) has published the most complete data, believe that the hypoglycaemia is only a terminal phenomenon. It has become evident that the true state of affairs in these infections is more complicated than Schern supposed, and in the field of carbohydrate metabolism difficulties of interpretation have increased rather than diminished as more investigators have reported their results.

Other blood changes have been much less thoroughly explored. Scheff (2) has published a report dealing with various aspects of the problem, beside the carbohydrate metabolism. He emphasizes the amount of oxygen consumed by the trypanosomes *in vivo*, a phenomenon which gives rise to an oxygen lack in the host, and leads eventually to death by "inner asphyxia." The upset in oxygen metabolism, the exhaustion of the free and reserve carbohydrates, and the probable presence of a toxin, determine the death of the host, in Scheff's opinion.

Scheff's data on the oxygen content of the blood could not be confirmed by Klinger, Geiger, and Comaroff (5). They found but little difference in this respect between normal and infected rats. Nor were the experiments which they devised to show a toxin successful. They incline to the view that the rapid multiplication of the parasites leads to the production of lactic acid in quantities which cannot be

successfully cared for by the host. The oxidative metabolism is thus upset, the alkali reserve depleted, and death ensues.

Discordant results have also been reported in regard to the liver glycogen. Scheff's data point to an exhaustion of this substance as the infection progresses, while Regendanz believes that the liver glycogen is always present in sufficient amount to keep the blood sugar at a normal level, and that the terminal lowering of the glucose is due to the action of a trypanosome toxin on the glycogen-glucose mechanism.

The strain of *T. equiperdum* was obtained from the Hygienic Laboratory in Washington. In rats it produces an acute septicaemic infection which terminates fatally in from 72 to 96 hours, after an incubation period which varies with the infecting dose. The majority of animals will not survive a concentration of trypanosomes much greater than one million per cubic millimeter of blood, although occasionally a concentration of nearly two million is observed. The rat does not show any resistance to the invader, so far as can be discovered. On the other hand, individual animals show marked variations in the number of parasites which they can tolerate before death ensues. Furthermore, the speed of reproduction as shown by the length of time in which the number of parasites doubles, shows considerable variation from rat to rat. Whether these are the results of individual resistance cannot be stated. It is clear, however, from the total counts, that the infection is by no means such a clear-cut, mathematically reproducible one as other workers have described for other strains.

Our present report deals with the foregoing matters and with certain other factors which have not yet been emphasized in trypanosome infections. These are points of attack by the parasites on the host's metabolism, the understanding of which is necessary for a complete picture of the disease. We have been interested in this infection also because of the information it may yield for the study of acute septicaemias in general. Infection with our strain of *Trypanosoma equiperdum* in the rat presents a relatively uncomplicated septicaemia: the infectious agents are confined nearly exclusively to the blood stream, while other tissues are involved to only a slight and irregular degree, and then only when the disease has almost run its course. Antibody production and leucocytic reactions are absent or evident to only a very slight degree; and the reticulo-endothelial system, the fundamental importance of which is well recognized in a large number of infections, is not involved (Dwijkoff (6)).

Methods

The CO₂ combining capacity, non-protein nitrogen, uric acid, and chloride determinations were made by the usual laboratory methods.* Micromethods were used for the other determinations, as follows: Liver glycogen—method of Slosse (7); blood sugar—method of Folin (8); lipid phosphorus—method of Krasnow and Rosen (9); cholesterol—method of Ling (10). Blood was obtained by heart puncture under light anaesthesia with iso-amyl-ethyl barbituric acid (amytal), approximately 7 mg. being given per 100 gm. of rat. In a few instances where the animals were *in extremis* anaesthesia was not used.

The data on the animals are arranged in the tables according to increasing numbers of trypanosomes per cubic millimeter of blood. It must be emphasized, however, that this arrangement shows only roughly the increasing severity of the disease. With this strain of trypanosomes, and with the stock of rats used, the lethal concentration varies greatly from one animal to another. For example, Rat 9, in Table II, with 925,000 organisms per cubic millimeter was moribund when bled, while Rat 16 in Table I, showing twice as many trypanosomes, still had several hours of life, to judge from its general appearance, breathing, activity, etc. This example is the most extreme one that occurs in our data, but it serves well to illustrate that the arrangement of figures shows a relative rather than an absolute severity of infection.

Carbon Dioxide Combining Capacity

In Table I are presented the data for the CO₂ combining capacities of 14 infected and 2 normal rats. Beginning with the fourth animal in the list (137,000 trypanosomes per cubic millimeter) all the infected rats show a well defined lowering of the CO₂ capacity. An acidosis is thus present from an early stage of the disease, varying in intensity from moderate to severe. The acidosis does not vary with the progress of the infection, as is shown by the figures for Rats 15 and 16, but drops while the number of trypanosomes is still small to varying levels below normal, and remains subnormal throughout.

Non-Protein Nitrogen, Uric Acid, Chlorides

The data on these constituents are presented in Table II, which gives the figures for 4 normal and 10 infected animals. Up to and including Rat 7 the figures fall within the normal ranges, but beginning with

* I wish to thank Mr. S. Graff, of the Department of Biochemistry, for these determinations, and Mr. R. Rebold, of the same department, for the sugar determinations on the hydrolyzed glycogen.

a higher concentration of trypanosomes (760,000 per cubic millimeter) there is a definite rise in the non-protein nitrogen and in the uric acid to figures which indicate pathological changes in the kidneys. These changes appear as preterminal rises, occurring at a time when 4 to 6 hours represents the estimated length of life.

Histologically the kidneys are normal until a late period in the disease. The changes which they show are those of an early nephrosis. The blood vessels are greatly swollen and in some areas hemor-

TABLE I

Carbon Dioxide Capacity of Serum in Rats Infected with Trypanosoma equiperdum

Rat No.	Trypanosomes per cu. mm.	CO ₂ capacity. Vol. per cent
1	Normal	59.5
2	Normal	62.0
3	75,000	65.0
4	137,500	37.0
5	200,000	13.6
6	225,000	45.0
7	275,000	35.0
8	525,000	25.0
9	820,000	47.8
10	825,000	36.0
11	830,000	31.9
12	850,000	20.2
13	1,000,000	31.5
14	1,125,000	14.5
15	1,175,000	46.0
16	1,850,000	40.4

rhages have occurred; the glomeruli are enlarged and fill the capsules; sections stained with Giemsa show them to be swarming with trypanosomes. They seem, however, to be intact, and undamaged, and the same is true of the collecting tubules. The secreting tubules, however, have undergone marked changes; they are swollen, and in some places the cells are broken down; fat has appeared in them, in the form of droplets, in large amounts, while it is absent in the glomeruli and in the collecting tubules. In some cases albumin is present in the urine.

The chlorides do not show any significant variations. In the three

TABLE II

Non-Protein Nitrogen, Uric Acid, and Chlorides in the Blood of Rats Infected with Trypanosoma equiperdum

Rat No.	Trypanosomes per cu. mm.	Non-protein nitrogen. Mg. per 100 cc.	Uric acid. Mg. per 100 cc.	Chlorides. Mg. per 100 cc.	
				Blood	Serum
1	Normal	39.6	2.8	482	590
2	Normal	33.0	3.0	461	592
3	Normal	29.0	1.9	453	—
4	Normal	32.0	2.6	468	—
5	475,000	37.0	2.9	470	—
6	525,000	36.0	3.1	432	573
7	600,000	36.4	2.7	465	575
8	760,000	42.0	4.8	472	—
9	925,000	138.0	11.3	381	—
10	1,050,000	73.0	7.8	420	566
11	1,100,000	47.0	5.6	459	572
12	1,275,000	57.0	5.6	453	—
13	1,450,000	56.0	6.1	470	—
14	1,800,000	40.0	3.9	—	—

TABLE III

Lipoid Phosphorus and Lecithin in Whole Blood of Rats Infected with Trypanosoma equiperdum

Rat No.	Before infection		After infection		Percentage increase	Trypanosomes per cu. mm.
	Lipoid P. Mg. per 100 cc.	Lecithin. Mg. per 100 cc.	Lipoid P. Mg. per 100 cc.	Lecithin. Mg. per 100 cc.		
1	8.05	201	10.35	258	28	25,000
2	7.05	176	9.22	230	30	292,000
3	9.25	231	10.00	250	8	300,000
4	8.9	222	10.85	271	21	575,000
5	8.1	202	11.75	293	42	575,000
6	8.9	222	10.8	270	21	600,000
7	9.6	240	12.5	312	30	625,000
8	7.8	195	10.55	253	35	740,000
9	8.8	220	13.3	332	51	850,000
10	9.05	226	11.6	290	28	1,200,000
11	8.8	220	17.5	437	98	1,800,000

instances in which they differ at all markedly (Rats 6, 9, and 10) they are lower than the normal range.

Lipoid Phosphorus and Lecithin

Table III contains the data for lipid phosphorus, and the calculated lecithin content in eleven animals before and after infection. Without exception the animals show increases in these constituents. The in-

TABLE IV

Cholesterol in Whole Blood in Rats Infected with Trypanosoma equiperdum

Rat No.	Cholesterol before infection. Mg. per 100 cc.	Cholesterol after infection. Mg. per 100 cc.	Trypanosomes per cu. mm.
1	76		
2	81		
3	68		
4	71		
5	87		
6	80		
7	86		
8	82		
9	80	74	717,000
10	74	76	750,000
11	67	67	820,000
12	76	85	830,000
13	73	69	850,000
14	76	78	900,000
15	74	84	1,250,000
16	76	83	1,275,000
17	66	69	1,850,000

crease begins very early in the course of the infection, when the parasites have been in the blood but a few hours (Rat 1), and has become nearly twice normal when the disease ends. With two exceptions (Rats 3 and 11) the increase varies between approximately 20 per cent and 50 per cent of the figure obtained from the normal animal.

Cholesterol

Cholesterol determinations are given in Table IV. The figures show that no changes in this blood constituent occur as the result of

the trypanosome infection. The amounts of cholesterol during the severe stages of the infection are all within the normal range, and vary but a few milligrams from the determinations before infection.

TABLE V

Liver Glycogen, as Glucose, and Blood Sugar, in Rats Infected with Trypanosoma equiperdum

Rat No.	Trypanosomes per cu. mm.	Grams of liver taken	Glucose. Mg. per gram of liver
1	Normal	1.162	20.1
2	Normal	1.117	16.3
3	87,500	1.043	0
4	100,000	1.269	0
5	112,500	2.085	3.7
6	137,000	1.064	6.0
7	225,000	1.108	3.4
8	300,000	1.198	7.0
9	500,000	0.688	0
10	660,000	2.426	0
11	825,000	1.172	0
12	1,000,000	1.081	0
13	1,075,000	2.098	0

Blood Sugar

Rat No.	Trypanosomes per cu. mm.	Blood sugar. Mg. per 100 cc.
1	112,500	67
2	292,000	112
3	300,000	141
4	575,000	79
5	660,000	88
6	740,000	111
7	1,200,000	75

Liver Glycogen and Blood Sugar

Determinations of liver glycogen and blood sugar are given in Table V. The two rats in the earliest stages of the infection were without glycogen demonstrable by the method used. The other rats which were positive had low glycogen values, compared with the controls. The last five animals, with counts of 500,000 trypanosomes per cubic

millimeter and over, did not show any glycogen, even when, as in two of the cases, over 2 gm. of liver were taken. Histologically, the livers of these animals show an enormous accumulation of fat.

DISCUSSION AND SUMMARY

Our results show that animals infected with *T. equiperdum* suffer from an acidosis that is present continuously from an early stage in the disease, and that this condition is complicated subterminally by the breaking down of the kidney structure. It is difficult to estimate how much influence the renal condition may have on the animal's death, but it is probably an important contributory cause.

The increase in lecithin may be of significance in relation to the disappearance of the liver glycogen and the maintenance of a normal blood sugar level. It is known that fat circulates in the blood as a lecithin-like compound (Bloor), and the production of sugar from fat, and of dextrose from glycerol (12), in the animal body, appears to be possible. For trypanosome infections, however, such statements can only be considered as suggestive possibilities, particularly in view of the fact that a hypoglycaemia develops while large amounts of fat are still present in the liver.

The absence of any change in the blood cholesterol in *T. equiperdum* infections is in contrast with the changes found for this constituent in various diseases such as typhoid fever, scarlet fever, and erysipelas; and in chronic infections such as malaria and syphilis. Our results also differ from those of Dubin (13) on one dog infected with this same organism. In this case, the infection was presumably a chronic one, and the dog had a cholesterol and lecithin blood content lower than normal. Our results for normal rats, using Ling's technique, are about 10 per cent lower than those reported by Randles and Knudson (14), who used Bloor's method.

SUMMARY

The CO₂ capacity of the serum is markedly lowered early in infection with *Trypanosoma equiperdum*.

The non-protein nitrogen and uric acid constituents of the blood are increased in the terminal stages. The kidneys also show terminal degenerative changes.

The cholesterol remains unchanged throughout.

Lecithin is markedly increased, most of the observations showing a 20 per cent to 50 per cent rise in this substance.

Liver glycogen is lower than normal in the early stages and could not be demonstrated in the later stages of the infection.

The blood sugar remains normal until a very late period in the disease.

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THE SURVIVAL OF YELLOW FEVER VIRUS IN CULTURES

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During the period from January to June, 1929, blood and tissues from many *rhesus* monkeys, experimentally infected with yellow fever, were planted in a variety of artificial media. Cultures were made, also, from several samples of human blood from patients with yellow fever; but these were prepared under less favorable circumstances than the cultures of monkey material, since there was usually a lapse of several days between the withdrawal of the sample and its arrival at the laboratory. The great majority of the cultures remained bacteriologically sterile, as determined by the absence of visible growth and our failure to find organisms by dark field examination and in stained smears.

Injection of Cultures into rhesus Monkeys

Cultures were injected into *Macacus rhesus*, and some of the inoculated animal subsequently had febrile temperatures; in a few instances it was possible to prove that yellow fever had really been contracted. In Table I a brief summary is given of nine experiments in which the proof was complete.

The longest period intervening between inoculation of culture medium and injection therewith of a test monkey, with resulting yellow fever, was 12 days. In this instance, McNeal-Marchoux medium had been employed, but the most consistently satisfactory results were obtained with a special egg medium. This was a modification of McCoy's egg-yolk medium (1) for the cultivation of *B. tularensis*. Its composition and mode of preparation were as follows:

Egg yolk..... 110 gm. (approximately)

Rabbit blood (defibrinated)..... 40 cc.

Distilled water..... 50 cc.

The ingredients were thoroughly mixed, coagulated in a slanted position in the Arnold sterilizer, and sterilized fractionally at a temperature of 75°C. In later

* This paper was prepared by Dr. N. C. Davis from the laboratory notes of Dr. Paul A. Lewis, who died of yellow fever in Bahia, Brazil, June 30, 1929.

TABLE I
Yellow Fever Infections in Monkeys Following Injection of Cultures Inoculated with Virus

Material cultured	Source of inoculum	Medium	Length of time in culture (days)	Quantity infectious blood injected (cc.)	Monkey inoculated	Strain of virus	Outcome	Criteria of infection
Liver	<i>Rhesus</i> H	Egg-Serum	2	0.25	<i>Rhesus</i> L	Asibi	Killed when moribund	Typical lesions in liver
Blood	<i>Rhesus</i> A	Egg-Serum	4		<i>Rhesus</i> B	Asibi	Killed on 2nd day of fever	Typical lesions in liver
Blood	<i>Rhesus</i> A	Egg-Serum	4 + 4*	?	<i>Rhesus</i> C	Asibi	Killed on 4th day of fever	Typical lesions in liver
Blood	<i>Rhesus</i> A	Egg-Serum	8	0.5±	<i>Rhesus</i> D	Asibi	Killed when moribund	Typical lesions in liver
Blood	<i>Rhesus</i> A	Marchoux	12	0.5±	<i>Rhesus</i> E	Asibi	Killed on 2nd day of fever	Positive transfers of blood. (Liver lesions not definite)
Blood	<i>Rhesus</i> B	Egg-Serum	9	0.2	<i>Rhesus</i> J	Asibi	Killed when moribund	Typical lesions in liver
Blood	<i>Rhesus</i> E	Egg-Serum	4	0.000033†	<i>Rhesus</i> K	Asibi	Killed when moribund	Typical lesions in liver
Blood	<i>Rhesus</i> U	Noguchi† (semi-solid)	7	0.1	<i>Rhesus</i> Z	S.R.	Fever. Recovered	Serum protected against virus
Blood	<i>Rhesus</i> U	Noguchi (semi-solid)	7	0.1	<i>Rhesus</i> AA	S.R.	Fever. Recovered	Positive transfer of blood. Animal afterward immune

* First condensation water from culture tubes injected into *Rhesus* B. Fluid replaced and injected into *Rhesus* C at end of second 4-day period.

† Culture also contained *Leptospirae*.

‡ Only condensation water and washings were injected; clot of original blood (diluted with normal blood) was not used.

experiments part of the egg yolk in this mixture was replaced by monkey liver and the medium was heated to 100°C. However, in the experiments in which the latter mixture was used, the results were negative.

A number of trials were made with the "MON" medium recommended by Kuczynski (2). It was impossible, however, to obtain the "normosal" called for in this formula, so a 1:5 dilution of sea-water was used instead. It is believed that none of the animals which were injected with cultures made on this medium contracted yellow fever. In one series the original quantity of blood was small (0.0001 cc.), but if the medium had been as favorable to the multiplication of the causative organism as its originator claimed, there should have been no trouble in securing an infection at the end of 4 days. In another series, the quantity of infectious blood in each tube was about 0.1 cc., but the incubation of the cultures lasted 7 days.

One series of experiments was made with Noguchi's semisolid *Leptospira* medium. Some of the inoculated tubes were left unsealed; the others were sealed with vaseline (partial anaerobiosis). None of the monkeys injected with these cultures developed yellow fever. In a second series, half of the tubes were inoculated with *Leptospira icteroides* (strain Palmeiras V) in addition to the infectious blood, since it was thought that the virus might maintain itself better in symbiosis with *Leptospirae*. At the end of 1 week mild infections were obtained by the injection of media both with and without *Leptospirae*; injections at the end of 2 weeks did not produce infection.

In a series of fifteen tubes of coagulated egg medium, the condensation fluid was supplemented by an egg-liver-brain extract with a trace of some substance for enrichment, either dextrose, lactose, maltose, sucrose, mannitol, glycerol, glycogen, peptone, or tryptic digest; and to this was added 0.001 cc. of infectious blood diluted to 0.05 cc. with normal monkey blood. No infections resulted from injections of this material at the end of 1 week.

The virus used in cultures represented three strains (Asibi, B.B., and S.R.) in routine use at the laboratory in Bahia (3). Except for the two cultures in semisolid medium with S.R. strain virus, all successful inoculations were with the Asibi strain. Cultures in Noguchi's medium were maintained at room temperature (22 to 25°C.); the other cultures were incubated at 35°C.

In addition to the experiments in which yellow fever was produced (see Table I), there were a number of others in which, without definite proof of infection, suggestive evidence was obtained.

The most interesting of these concerned *Rhesus Q*, which was injected with material from a culture on egg medium 18 days old. The animal had fever on the second and third days following injection; the maximum temperature was 105.2°F. 16 days after the first injection he was given an immunity test with fresh infectious blood and again had fever, this time for 6 days, with a maximum temperature of 104.6°F. Recovery followed.

Injections of Dil.

Quantity of blood (cc.)	Experiment I. May 28, 1929			Experiment II. June 7, 1929		
	Strain of virus	Animal inoculated	Outcome	Strain of virus	Animal inoculated	Outcome
3.0						
1.0	Asibi	<i>Rhesus F</i>	Killed on 2nd day of fever. Lesions in- definite			
0.1	Asibi	<i>Rhesus G</i>	Fever. Dead on 4th day. Typical yellow fever			
0.01	Asibi	<i>Rhesus H</i>	Fever. Killed when moribund on 4th day. Typical yellow fever			
0.001	Asibi	<i>Rhesus I</i>	Fever. Killed when moribund on 5th day. Typical yellow fever	Asibi	<i>Rhesus P</i>	Fever for 5 days. covered
0.0001				Asibi	<i>Rhesus O</i>	Fever. Died on Typical yellow
0.00001				Asibi	<i>Rhesus N</i>	Fever. Killed moribund on Typical yellow
0.000001				Asibi	<i>Rhesus M</i>	No fever. Died day. Probable fever

Infectious Blood

Experiment III. June 11-14, 1929			Experiment IV. June 20-24, 1929		
Strain of virus	Animal inoculated	Outcome	Strain of virus	Animal inoculated	Outcome
S.R.	<i>Rhesus S</i>	Fever. Killed when moribund on 4th day. Typical yellow fever			
S.R.	<i>Rhesus U</i>	Fever. Killed when moribund on 6th day. Typical yellow fever	S.R.	<i>Rhesus BB</i>	No fever. No reaction to immunity test
S.R.	<i>Rhesus R</i>	Fever. Killed when moribund on 5th day. Typical yellow fever	S.R.	<i>Rhesus Y</i>	Killed on 2nd day of fever. Typical yellow fever
S.R.	<i>Rhesus T</i>	Fever. Killed when moribund on 5th day. Typical yellow fever	S.R.	<i>Rhesus X</i>	Fever. Killed when moribund on 6th day of fever. Typical yellow fever
			S.R.	<i>Rhesus W</i>	No fever. No reaction to immunity test
			S.R.	<i>Rhesus V</i>	Fever. Killed when moribund on 7th day. Typical yellow fever

Many animals that showed no temperature reaction to the original injection later proved partly or wholly refractory to test doses of virus; the assumption is that some immunity was called forth by the dead or attenuated virus present in the cultures.

Rhesus B was inoculated with the condensation water from two culture tubes at the end of 4 days' incubation, containing in all about 0.25 cc. of infectious blood. The fluid from these tubes was replaced with that from uninoculated medium, and incubation was continued for 4 days more. *M. rhesus C* was inoculated with the fluid from these same tubes at the end of the second 4-day period. Although *Rhesus B* and *Rhesus C* were sacrificed while still in the febrile stage, both had liver lesions typical of yellow fever. It was thought at first that there might have been reproduction of the virus in the culture medium, but the results of dilution experiments suggested that probably there was merely a survival from the original inoculum.

Injections of Infectious Blood Highly Diluted

A summary of the dilution experiments is given in Table II. It will be noted that while Experiment III and Experiment IV each represents a single series of dilutions, in neither case were all injections made on the same day or with blood from the same monkey. Each of these experiments might be subdivided, making six in all, except that additional space would be needed for recording them and the results are sufficiently clear in the present scheme. Dilutions of infectious blood were made in every case with either citrated or defibrinated blood from normal monkeys. Injections were made intraperitoneally.

It is evident that both the Asibi and S.R. strains of virus were fatal in quantities of 0.00001 cc. of infectious blood. There is no reasonable doubt that the monkey which received 0.000001 cc. of blood containing Asibi strain virus also succumbed to yellow fever. The liver section showed a considerable number of postmortem changes, but there was undoubtedly a severe injury preceding death. Aragão (4) records positive results from the injection of a millionth part of an infected mosquito.

DISCUSSION

It has been shown by Sawyer, Lloyd, and Kitchen (5) that citrated or clotted blood from animals infected with yellow fever retains

a certain amount of virulence after storage for at least 35 days and that glycerinated blood is capable of infecting animals after preservation for 60 days. Their specimens were kept at refrigerator temperature. The dosage which produced yellow fever was 1 cc.

From the results of the dilution experiments recorded above, it would seem certain that the infections obtained from the injection of cultures were due to the survival of virus from the original inoculum. The only surprising point is that virus survived in infective quantities for at least 12 days at a temperature of 35°C., when usually it dies out quite rapidly in citrated blood at room temperature.

It is believed that such of the infections reported by Kuczynski (2) as were actually yellow fever, were caused by virus which survived in the medium and not by the visible organisms under cultivation. Since extremely minute quantities of virus will produce infection, it is easy to account for the virulence of subcultures. This explanation does not preclude the possibility that the bacteria of Kuczynski, although probably not the cause of yellow fever, may have had some pathogenic properties of their own.

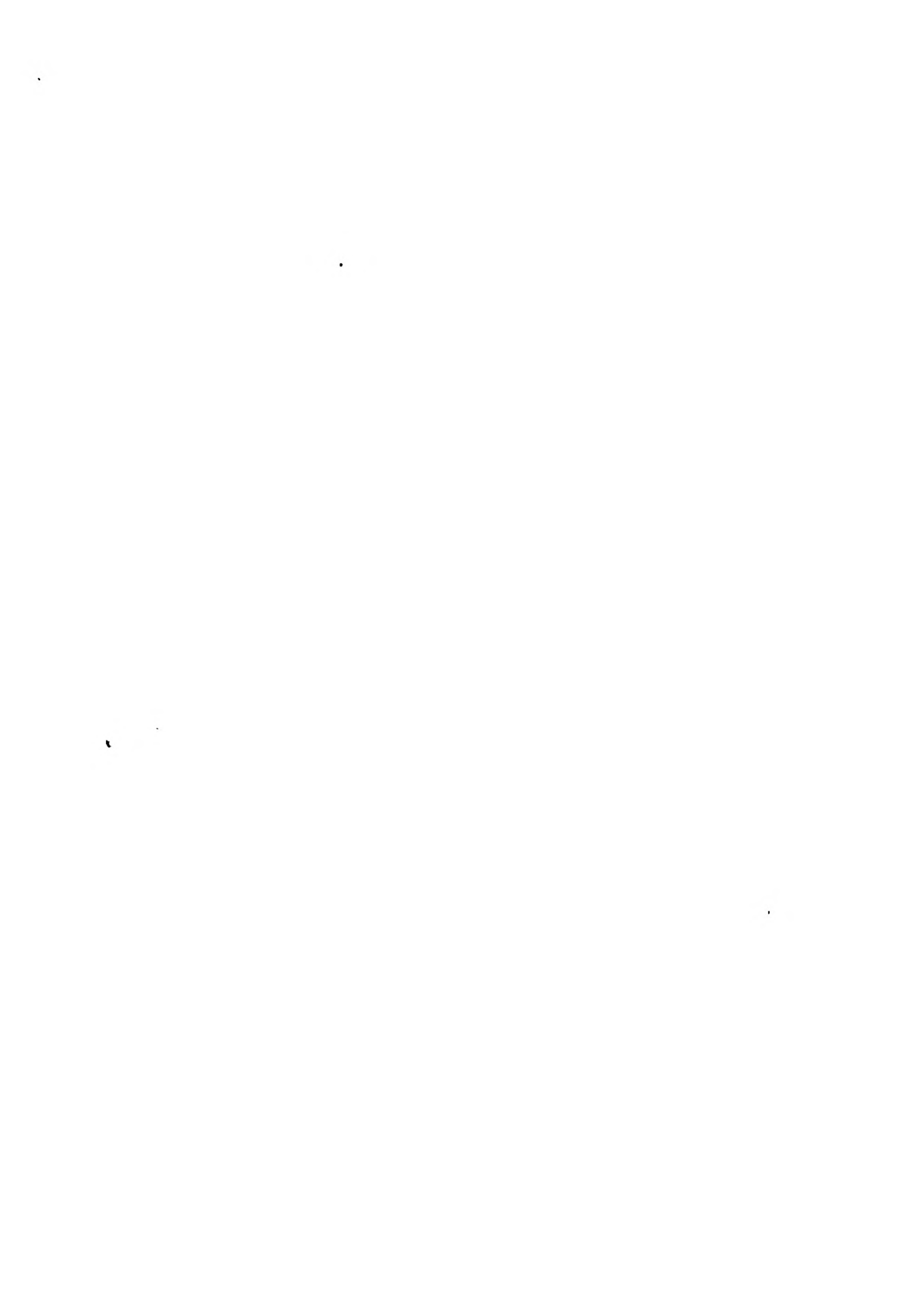
SUMMARY AND CONCLUSIONS

1. The virus of yellow fever has been found to survive in artificial culture media for at least 12 days at a temperature of 35°C. No visible growth has been present and no reproduction of the virus has been demonstrated.

2. Infections have been obtained in *rhesus* monkeys with two strains of virus in quantities as small as 0.00001 cc. of infectious blood, and with one strain in an amount probably as minute as 0.000001 cc.

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STUDIES ON BARTONELLA MURIS ANEMIA OF ALBINO RATS*

I. TRYPANOSOMA LEWISI INFECTION IN NORMAL ALBINO RATS ASSOCIATED WITH BARTONELLA MURIS ANEMIA

II. LATENT INFECTION IN ADULT NORMAL RATS

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It has been observed (1, 2) that removal of the spleen in adult albino rats is followed by a severe anemia within a few days to 3 weeks. This anemia is characterized by a marked drop in the normal red cell count from 8 to 10 million cells per cubic millimeter to 1 million or less. There is a corresponding drop in the hemoglobin and the appearance in the red blood cells of inclusion bodies known as *Bartonella muris* bodies. These bodies appear as diplococci and rods on the surface of the cell. They occur in very large numbers (20 to 30 per cell) at the height of the anemia. Poikilocytes, anisocytosis, polychromasia and normoblasts are present in the circulating blood. Erythrocytosis by large circulating mononuclears is occasionally observed. Noguchi has succeeded in growing a bacillus for one generation from the blood of infected rats on a special *Leptospira* medium, to which blood has been added (3). The failure of other investigators to isolate these organisms has cast some doubt on the etiological rôle played by these bodies in the anemia (4). Nevertheless their persistent appearance coincidental with the anemia is striking.

All the rats used in our studies have been raised in our laboratory from original Wistar Institute stock. The anemia develops in the adult rats of this stock following splenectomy in all instances. About 20 per cent of the rats die within 8 to 10

* Read before the American Association of Pathologists and Bacteriologists, April 17, 1930.

days from anemia. The anemia begins as early as the 3rd day and as late as the 16th day. It is accompanied by a severe hematuria. The disease is rapidly progressive, reaches its peak about the 8th to the 12th day and then diminishes. In those recovering from the disease it disappears after 3 to 5 weeks, the count returning to the normal level. Recurrences occasionally occur, but if the rat survives the height of the first attack it survives subsequent attacks. At times the onset is precipitous, the number of red blood cells per cubic millimeter dropping to 2 or 3 million within 24 hours after the onset. Males develop the anemia more rapidly than females. It has been observed that some strains of rats do not develop the anemia (5).

Autopsy reveals a profound anemia of the organs and marked icteric tint to the subcutaneous fat. There is generally free fluid in the serous cavities, the blood in the heart is unclotted and watery. The liver, kidneys and heart show marked fatty changes. The bone marrow is red. Microscopically there are focal areas of necrosis in the liver. The Kupfer cells are markedly distended with engulfed red blood cells, hemosiderin pigment and *Bartonella muris* bodies. In general the reticulo-endothelial system is severely damaged. The kidneys reveal an extensive nephrosis and in some instances embolic hemorrhagic glomerular nephritis. The glomeruli may show necrotic changes, the tubules show marked degeneration and their lumina are filled with albuminous material and iron containing pigment and occasional casts of red blood cells. There may be small areas of infarction in the kidneys without any surrounding inflammatory response. This is most striking in young rats with a fatal infection. In very anemic young rats with intact spleens, the pulp cells show extensive degenerative changes and there are areas of necrosis.

From the work of Lauda (1) and Mayer (2) the infectious nature of the anemia had been suspected, but the anemia could not be transmitted from the splenectomized rat to any adult normal animal. Ford and Eliot (4) were able to transmit this anemia through many passages in immature normal rats under 40 gm. in weight and immature normal rabbits by direct injection of whole blood of a splenectomized anemic rat. This would indicate a difference in the functional activity of the spleen in the young and adult animal. It would suggest that the adult normal rat is resistant to the anemia due to a permanent immunity from an infection with the "virus" of *Bartonella muris* anemia in early life. This immunity is broken down in the adult rat by the removal of the spleen and a second invasion with the virus occurs. It is possible, however, that the virus remains latent in the adult rat following an early infection and that the adult normal rat though immune is a "carrier."

An effort was made to determine experimentally (a) whether the adult normal rat is a "carrier" of the virus of the anemia; (b) whether injury to the spleen by a protozoan infection permits the occurrence of an anemia and if so, whether such an anemia is identical with the *Bartonella muris* anemia.

1. The Transmission of a Strain of the *Bartonella muris* from Splenectomized Adult Rats to Normal Young Rats and Rabbits

The anemia of splenectomized rats may be transmitted to young rats under 40 gm. and to 3 week old rabbits. This observation made by Ford and Eliot has been confirmed by us.

The injection of 0.5 cc. of whole blood from a splenectomized rat intravenously into 3 week old rabbits is followed by a profound anemia within 10 days which persists for several days and within 3 weeks the blood count returns to normal. The anemia can then be transmitted to baby rats for many transfers.

Sample Protocol.—Rabbit 4 injected intravenously with 0.5 cc. of whole blood from a splenectomized rat at height of the anemia.

	Blood count	Hbg. (Dare)	Bert. bodies
* 1/16—Before injection	6,350,000	105%	
1/20—After injection	6,600,000	90%	
1/22 " "	3,900,000	37%	Numerous rods and diplococci
1/25 " "	1,800,000	10%	"
1/28 " "	1,850,000	50%	"
1/30 " "	3,500,000	60%	
2/8 " "	5,310,000	90%	Occasional rods and diplococci

* Injected same day with blood of anemic rat.

The hemoglobin drops more rapidly than the red cell count and returns more rapidly to normal.

2. *Trypanosoma lewisi* Infection Associated with *Bartonella muris* Anemia in Normal Albino Rats

Mayer (7) noted the occasional appearance of *Bartonella muris* rods in the cells of mice and rats infected with trypanosomes.

In studies on *Trypanosoma lewisi* infection in adult albino rats we observed a moderate anemia during the first week of infection, with

the appearance in small numbers of rods and diplococci of the *Bartonella muris* bodies in the red cells. An effort was made to determine whether this anemia found in trypanosome infected rats is identical with the *Bartonella muris* anemia of splenectomized rats.

0.5 cc. of blood of an albino rat infected with *Trypanosoma lewisi* was injected into 3 week old rabbits. The trypanosomes died in the rabbit but a moderate anemia occurred within 10 days with the appearance of rods in the red cells. A small quantity of the blood of the anemic rabbit was injected into 30 gm. rats and an anemia developed in these rats with the appearance of the rods and cocci in the red cells. The virus of the anemia was thus transmitted through 30 gm. rats for several transfers. In this manner a strain of the virus of *Bartonella muris* anemia was isolated from adult nonsplenectomized albino rats infected with *Trypanosoma lewisi*.

Trypanosoma lewisi infection in adult albino rats causes a profound disturbance in the function of the spleen and lowers the resistance of the rat to the virus of the *Bartonella muris* anemia. The deaths sometimes observed in young rats from *Trypanosoma lewisi* are due to the concomitant *Bartonella muris* anemia which in young rats is so often fatal. *Trypanosoma lewisi* infection causes a marked increase in the size of the spleen. The pulp cells become engorged with engulfed red blood cells and a marked disturbance in the function of these cellular elements is present. The effect is similar to that of splenectomy in the adult rat.

Latent Bartonella muris Anemia in the Adult Albino Rat

It has been observed that the *Bartonella muris* anemia cannot be transmitted to adult animals with intact spleens by direct injection of blood from an infected animal. This infection can be transmitted, however, to normal young rats with intact spleens. This would indicate the probability of an infection early in life which results in a permanent immunity to the infection in the rat. This immunity is broken down with removal of the spleen and permits a second invasion with the virus. The rat often recovers, but repeated attacks may be produced either by the injection of whole blood from other anemic splenectomized rats or may occur spontaneously. It is possible however that the virus of anemia is present in the system from an infection in early life, and remains latent in the adult albino rat. An effort was

*The Isolation of Bartonella muris Virus from Trypanosome Infected Unoperated Adult Rats
and from Normal Unoperated Adult Rats (Sample Protocols)*

The letter "T" is used in place of the last three zeros in the red cell count. The hemoglobin is expressed in percentages as calculated from Dare hemoglobinometer readings.

Adult rats	3 week rabbits	30 gm. rats
<i>Unoperated Normal Rat</i> → <i>Rabbit 2</i>		
8,000 T	5,600 T 95%*	
	3,600 T 50% → 11E 6,300 T 90%	16N 6,200 T
		18R 5,400 T 90% → 31R (Strain NS) 6,400 T 95%
		3,400 T 50%
		19R 5,750 T 95%
		4,000 T 60%
		17N 8,000 T
		2,200 T
<i>Unoperated Normal Rat with Trypanosoma lewisi Infection</i> → <i>Rabbit 1</i>		
9,300 T	5,700 T 90%	14R 5,600 T
4,450 T		3,000 T
	8D 6,140 T 90%	20R 5,000 T 95%
	2,800 T 50%	3,300 T 50%
	5,700 T 90% → 9D 5,160 T 90%	21R 5,200 T 100% → 30R (Strain TS) 5,150 T 95%
		3,400 T 50%
		3,300 T 50%
	2,450 T 50%	15R 6,500 T
		3,450 T
		6C 5,400 T 85%
		3,810 T 60%
		24R 5,500 T
		3,250 T
<i>Splenectomized Rat</i> → <i>Rabbit 4</i>		
9,700 T 100%	6,600 T 100%	25R 5,400 T → 32R (Strain SP-4) 6,400 T
965 T 10%		2,400 T
	1,800 T 10%	3,150 T

All the young rats showed *Bartonella muris* bodies in the red cells after injection with blood of the other animals.

* Only the red blood cell count before injection and lowest count after injection indicated.

made to determine whether or not a latent infection with the *Bartonella muris* virus is present in the normal adult unoperated rat.

Repeated examination of smears of the blood of normal rats failed to reveal the presence of the *Bartonella muris* bodies in the red cells. It was found that intravenous injection of 0.5 cc. of the blood of a normal adult rat into young rabbits produced anemia with the appearance of the *Bartonella muris* bodies in the red cells. The transfer of 0.5 cc. of the blood of anemic young rabbits intraperitoneally into young rats is followed by a severe anemia in these rats with the appearance of *Bartonella* bodies in the red cells. The anemia can be passed through successive transfers in young rats. The blood of a normal rabbit produces no effect when injected into young rats. The blood either of young or of mature normal rats produces no effect when injected into other young rats.

By the passage of whole blood of normal adult rats through young immature rabbits the virulence of the virus of the latent infection with the *Bartonella* anemia is sufficiently enhanced to produce the infectious anemia in young rats. It may be that the virus increases in quantity in the rabbit sufficiently to produce an anemia when the blood is subsequently injected into the rat.

The Effect of Splenectomy in Young Rats

The adult normal rat is a carrier of the *Bartonella muris*. Further efforts were made to determine at what time in the life cycle of the rat it becomes a carrier of the virus of the *Bartonella muris* anemia.

Six 3 week old suckling rats were splenectomized. These young rats were placed in sterilized cages, completely isolated from the stock rats. None of these rats developed *Bartonella muris* anemia. It is evident then that during the first weeks of life the rat does not harbor the virus of *Bartonella muris* anemia, and therefore removal of its spleen is not followed by the infectious anemia. Since this period corresponds to the only period of life during which the *Bartonella muris* anemia may be transmitted in normal young rats by the injection of blood from an anemic splenectomized adult rat, it is not probable that the failure of splenectomized young rats to develop spontaneously the *Bartonella muris* anemia is due to a temporary protective immunity conferred from the mother. It would further suggest that the transmission of the virus from rat to rat is not necessarily through an insect host.

These experiments indicate that the *Bartonella muris* infection is latent in the adult normal rat. These rats are carriers of the virus and from their blood the virus can be isolated by passage through young rabbits. The invasion of the rat with this virus must occur after the suckling period, since suckling rats do not harbor the virus. The experiments further suggest a difference in the function of the spleen in young and adult rats.

DISCUSSION

From the data presented it can be concluded that the adult rat is a carrier of the virus of *Bartonella muris* anemia. The failure to produce the picture of *Bartonella muris* anemia in other adult animals by removal of the spleen may be due to the fact that these animals are not carriers of the virus. The normal adult rat cannot be given the anemia by direct injection of blood from an anemic splenectomized rat, since the adult rat possesses a high degree of resistance, presumably from a transient infection in early life. During the suckling period the young rat does not harbor the virus of the anemia. Therefore removal of the spleen in these rats is not followed by *Bartonella muris* infection.

The spleen is physiologically and anatomically injured in many protozoan infections as in trypanosomiasis, leishmaniasis, spirochetosis and piroplasmic diseases and malaria. The spleen plays an important rôle in resistance of the organism to *Bartonella* infections. Severe injury to the spleen may bring about the same condition as splenectomy. In many mammals *Bartonella* and *Grahamella* infections accompany protozoan infections. It has been suggested by Bayon (8) that the *Grahamella* is capable of invading the organism as a result of injury to the spleen by a hematozoic infection. *Grahamella* infections are not observed in rodents under other conditions. From our work on *Bartonella muris* infection in rats, however, it is evident that the *Bartonella* anemia in the adult rat occurs only after an immunity to this infection acquired in early life is broken down by injury to or removal of the spleen. The spleen is the important factor in the maintenance of the resistance. No doubt *Grahamella* infections occur under similar circumstances.

Bartonella bacilliformis infection in man is a disease resembling the *Bartonella* and *Grahamella* infections of rodents. Oroya fever is a systemic infection charac-

terized by profound anemia and the occurrence of *Bartonella* bodies in the red cells. Verruga peruana is a skin infection without anemia and in this no *Bartonella* bodies occur in the red cells but they are found in the endothelial cells in the skin lesion. Noguchi (9) and Mayer and Kikuth (10) have demonstrated the common etiological factor in these two human diseases. It is of importance to determine what factors in the individual are responsible for the occurrence of one type or the other. From the observations in the literature in analogous infections and from our own work with *Bartonella muris*, an explanation presents itself. Verruga peruana may be a first infection in an individual with a normal spleen. Oroya fever may be a recurrence of an early infection or a flaring up of a latent infection in an individual with an injured spleen. Chronic malaria is a very common concomitant infection in individuals with Oroya fever. Such a chronic protozoan infection causes profound physiological injury to the spleen and may thus be responsible for the breaking down of the resistance of the individual to the *Bartonella* infection. Noguchi (11) made an effort to demonstrate the effect, if any, of malaria and of splenectomy on the course of infection with *Bartonella bacilliformis* in monkeys. From several observations he concluded that both malaria and *Bartonella bacilliformis* may coexist without unfavorable effect of one disease upon the course of the other, that splenectomy led to no appreciable aggravation of the *Bartonella* infection and that both malaria and splenectomy in the same animal had no appreciable effect on experimental verruga infection. The experiments, however, were not comprehensive enough to establish the relationship of chronic malarial infection to Oroya fever, nor do they disprove the conception that the spleen plays an important rôle in the latter disease.

Suggestive is the single observation made by Noguchi on a chimpanzee infected with *Bartonella bacilliformis*. A severe anemia developed in this animal only after an accidental infection with Rocky Mountain fever by the bite of the *Derma-centa andersonii* and disappeared only when the blood became negative on culture.

Kikuth (12) reports an increase in the number of "takes" of verruga peruana from 50 to 100 per cent following splenectomy in monkeys inoculated with material from human verruga papulae. In one instance typical Oroya fever with severe anemia developed in one of the monkeys that previously had been injected with verruga material but that had failed to develop lesions and subsequently was reinjected with such material.

These observations and our own in *Bartonella* infections in rats offer a possible explanation of the modus operandi of *Bartonella* infections in human beings. A first infection with *Bartonella bacilliformis* may result in verruga peruana or may have no clinical manifestations. Subsequent injury to the spleen by a chronic protozoan infection breaks down the protective mechanism of immunity to this organism with the occurrence of a systemic infection with the *Bartonella bacilliformis* in Oroya fever.

SUMMARY

1. The virus of *Bartonella muris* anemia of splenectomized rats may be transmitted to normal young unoperated rats and rabbits. This confirms the observations of Ford and Eliot.

2. *Trypanosoma lewisi* infections in normal adult rats are accompanied by an anemia most marked at the height of the infection and the appearance of *Bartonella muris* bodies in the red blood cells.

3. In young rats *Trypanosoma lewisi* may produce death from the severity of the anemia, complicating the disease. The anemic virus may be separated from the *Trypanosoma lewisi* infected blood by passage through young rabbits with subsequent maintenance of the strain in immature rats.

4. A strain of the virus of *Bartonella muris* anemia capable of producing an anemia in young rabbits and young rats for successive transfers has been isolated from the blood of normal adult unoperated rats by passage through young rabbits.

5. The adult normal rat is a carrier of the *Bartonella muris* virus.

6. Splenectomy in young suckling rats separated from the mother is not followed by a *Bartonella muris* anemia. The young suckling rat is not a carrier of the infection.

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STUDIES ON BARTONELLA MURIS ANEMIA OF ALBINO RATS*

III. THE PROTECTIVE EFFECT OF AUTOPLASTIC SPLENIC TRANSPLANTS ON THE BARTONELLA MURIS ANEMIA OF SPLENECTOMIZED RATS

By DAVID PERLA, M.D., AND J. MARMORSTON-GOTTESMAN, M.D.

(From the Laboratory Division, Montefiore Hospital, New York)

PLATE 6

(Received for publication, April 24, 1930)

Removal of the spleen in the adult albino rat is followed within 6 to 10 days by a severe progressive anemia which is fatal in 20 to 25 per cent of rats (1, 2, 3). In our laboratory approximately 100 per cent of splenectomized rats develop this anemia. Unsuccessful attempts have been made by investigators to protect rats from the anemia following splenectomy by feeding or injecting splenic tissue (Ford (4)) and by feeding large amounts of liver tissue (5). Vedder (5) attempted to transplant portions of spleen into the tunica vaginalis of the testes but was unsuccessful.

The first successful autotransplantation of splenic tissue in animals was made by Marine and Manley (6). They found that subcutaneous or intramuscular autoplasmic spleen transplants in the rabbit are successful in almost 100 per cent of instances. Age is an important factor in the growth of the transplant. The younger the rabbit, the more rapid the growth of the transplant. Removal of the spleen is a powerful stimulus to the growth of the transplants. In mature rabbits the transplants took but usually did not grow and were often slowly resorbed. From their observations they concluded that in the rabbit the spleen is most important in early life. After sexual maturity it is either unimportant or its function may readily be assumed by other tissues (hematopoietic).

They have demonstrated that whereas anatomically the spleen is a highly complex structure, biologically, all the major elements of the spleen are simple as indicated by the uniform and marked regenerative capacity of their transplants.

The experiments reported in this communication were undertaken to determine: (a) Whether autoplasmic transplants of splenic tissue

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The experiments reported in this communication were undertaken to determine: (a) Whether autoplasmic transplants of splenic tissue

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in the adult rat regenerate, (b) what protective effect such autoplasmic transplants of splenic tissue would have against the *Bartonella muris* anemia, and (c) the minimum amount of spleen left *in situ* that would protect the rats against this anemia.

The experiments were divided into three groups. In the first group autoplasmic transplants of small pieces of splenic tissue were made in the abdominal wall of eight rats and 4 weeks later the spleen was removed. Three splenectomized rats in which no transplants had been made were used as controls.

Methods

In this work mature albino rats about 3 months of age were used. These rats were raised in our laboratory from Wistar Institute stock. They were approximately uniform in weight. The methods for preparation of the animals for transplantation have been described in a previous communication (7).

The spleen was exposed through a small incision in the abdominal wall and one pole was gently pulled up into the opening. A piece was cut off for transplantation. Pieces of spleen about 6 mm. in diameter were immediately placed in small prepared pockets in the abdominal wall. Great care was exercised to avoid hemorrhage into these pockets and if hemorrhage occurred, a new pocket was made. The sites of the transplants were indicated by the closing of the mouth of the pocket with a black silk suture. Four transplants were usually inserted into each animal. 4 weeks later the abdominal cavity was again opened, the mesentery of the spleen ligated and the spleen removed. The peritoneum and skin were closed. Red blood cell counts and hemoglobin determinations were made at frequent intervals.

The results of this experiment (tabulated in Table I) indicate that transplants made 4 weeks before splenectomy afford no protection against *Bartonella muris* anemia. Histological examination of the transplants of Rat 1082 which died at the height of the anemia revealed replacement of the plant by reticular cells that formed large sinusoid spaces filled with red cells. Degenerative changes of the reticular elements and areas of necrosis were present. The splenic tissue, both pulp and follicles, had not regenerated at this time. The reticulum present, however, showed those same changes of degeneration and focal necrosis found in the spleen of young rats with *Bartonella muris* anemia. It is of interest that the transplant though not regenerated, reacted to the infection in the same way as the intact spleen. Marine and Manley (7) observed that in rabbits dying of pneumonia the

spleen transplants on section were soft, engorged with blood and microscopically showed an increase in pulp cells. These changes are the same as those found in the intact spleen in the presence of infection. Five rats were not killed until 3 months after the transplants had been made. Upon examination the transplants were very large in size. It is evident that complete regeneration of both the pulp cells and Malpighian corpuscles of the transplants had occurred within 3 months. In the absence of the spleen there was a marked hypertrophy of the regenerated transplants. These transplants were really small spleens. They were not regenerated, however, at the time the spleen was removed and did not prevent the development of *Bartonella muris* anemia (Table I).

In the second group of experiments nine adult albino rats were used. In seven of these, autoplasmic splenic transplants were made 7 weeks prior to splenectomy. In three rats the spleen alone was removed.

The method of transplantation has been described above. In these rats two transplants were inserted on either side of the midline in the sheath of the rectus muscle. Seven weeks later the spleen was removed.

In this group four of the seven rats with transplants did not develop an anemia and failed to show *Bartonella muris* bodies in the red cells. More than half the rats were protected by autoplasmic spleen transplants. Three weeks after the splenectomy or 10 weeks after the transplantation the rats were killed with ether and the transplants carefully removed and sectioned (see Table II).

Comparative histological studies of the transplants in the protected and unprotected rats reveal a striking contrast. In those rats that were unprotected the Malpighian corpuscles of the transplants are completely regenerated, but the entire pulp is filled with red blood cells. The corpuscles stand out strikingly against the surrounding intense congestion (Fig. 2). Large sinusoids are present but are distended with blood and the endothelium flattened. In those rats that were protected the transplants are smaller, but both the pulp cells and the Malpighian corpuscles are completely regenerated and have the appearance of adult splenic tissue (Fig. 3). In the transplants that protected, large amounts of hemosiderin pigment were present in the

Effect of Spleen Autotransplants Made 4

No. and sex	Weight gm.	Date of trans- plant	Interval between transplant and splenectomy	Blood count before splenectomy	Counts after splenectomy					
					1	3	5	6	7	9
1078 M	215	10/6	4 weeks	90% 9,600 T	80% 8,900 T	81% 8,350 T	45% 3,550 T	44% 4,150 T	45% 4,100 T	60% 5,050 T
1079 M	203	"	"	95% 9,600 T	85% 8,550 T	78% 9,800 T	Died			
1080 M	230	"	"	90% 8,500 T	95% 9,500 T	90% 9,450 T	28% 1,450 T	34% 3,900 T	25% 4,100 T	42% 3,550 T
1081 M	203	"	"	90% 9,300 T	81% 7,500 T	85% 8,250 T	32% 4,100 T	26% 2,400 T	35% 3,700 T	50% 4,400 T
1082 M	203	"	"	100% 11,200 T	75% 9,000 T	75% 7,950 T	40% 4,900 T	Died		

The letter "T" is used in place of the last three zeros in the red cell count. The hem

• Splenectomy on *Bartonella muris* Anemia

days								1/23/30
16	20	24	27	31	37	45	55	Histology of transplants
80% 7,500 T	90% 8,900 T	90% 8,650 T						Fully regenerated splenic tissue with prominent follicles and normal pulp tissue. Pigment in endothelial cells
75% 4,800 T	70% 4,600 T	8,150 T	80% 7,150 T	6,800 T	4,000 T	4,200 T	4,800 T	Small spleen with prominent Malpighian corpuscles and congested pulp. Erythrophagocytosis pulp cells present. Sinuses seem to be present and filled with red blood cells
								11/9/29 Malpighian corpuscles are not present. Pigment in cells of capsule. Transplant replaced by reticulum showing marked swelling and necrosis of cells with occasional cell containing pigment. Small foci of necrosis are present as are seen in spleen of young anemia rats

ssed in percentages as calculated by the Dare hemoglobinometer.

No. and sex	Weight	Date of transplant	Interval between transplant and splenectomy	Blood count before splenectomy	Counts after splenectomy					
					1	3	5	6	7	9
	gm.									
1083 F	189	10/6	4 weeks	98% 9,800 T	72% 7,500 T	70% 6,150 T	15% 3,300 T	12% 1,900 T	10% 2,100 T	Died
1084 F	179	"	"	98% 9,750 T	82% 8,750 T	78% 9,750 T	20% 2,370 T	22% 2,150 T	10% 2,050 T	18% 2,150 T
1085 F	170	"	"	93% 10,010 T	85% 9,200 T	87% 7,200 T	39% 2,800 T	30% 2,800 T	38% 2,500 T	42% 4,500 T
1086 M	205	None	Control splenec.	96% 10,000 T	89% 7,300 T	85% 7,225 T	48% 3,600 T	26% 1,700 T	15% 2,300 T	Died
1087 M	217	"	"	94% 9,800 T	67% 9,650 T	69% 7,000 T	78% 5,850 T	88% 6,950 T	45% 6,100 T	Died
1088 F	180	"	"	90% 10,030 T	87% 5,800 T	80% 6,500 T	35% 5,000 T	23% 2,700 T	18% 1,700 T	22% 2,650 T
1089 F	162	Normal control		90% 8,000 T	95% 8,550 T	100% 8,450 T	95% 9,600 T	100% 9,550 T	85% 7,000 T	85% 8,150 T

reticular cells of the pulp. In those that did not protect, though there was so striking a quantity of blood present and though the rats had had severe anemia, no hemosiderin pigment was deposited in the few cells of the pulp. The reticular cells of the splenic pulp had undergone an exhaustion destruction that exceeded the rate of regeneration.

d

f days								1/23/30
16	20	24	27	34	37	45	55	Histology of transplants
75% 3,900 T	70% 4,100 T	4,800 T		80% 7,150 T	6,000 T	80% 6,100 T	75% 6,600 T	Malpighian corpuscles prominent, stand out against intense congestion about them in pulp. Endothelial and reticular elements as found in normal spleen are seen
80% 6,300 T	85% 6,500 T	6,600 T		6,700 T	6,250 T	6,150 T	6,200 T	Malpighian corpuscles prominent. Pulp intensely congested. Erythrophag prominent. Pigment present
70% 4,550 T	5,100 T	8,600 T	75% 6,450 T	5,500 T	5,650 T	5,300 T	5,900 T	
85% 8,600 T	85% 8,750 T	7,200 T	8,200 T	7,500 T	7,800 T	7,500 T	8,600 T	

In the third group, an effort was made to determine the minimum amount of spleen left *in situ*, that would prevent the development of the anemia. Eight adult rats were used. In two rats one-quarter of the spleen was removed, in two one-half of the spleen was removed, in two three-quarters of the spleen was removed and in two the entire spleen was removed.

No. and sex	Weight	Date of transplant	Interval between transplant and splenectomy	Count before splene- ctomy	Counts after splenectomy--					
					1	3	4	5	6	8
	gm.									
1204 F	110	11/23/29	7 weeks	108% 8,500 T	105% 8,500 T	95% 8,000 T	108% 7,300 T	110% 8,000 T	100% 9,100 T	100% 8,310 T
1205 F	133	"	"	110% 8,700 T	110% 8,500 T	95% 8,200 T	103% 8,160 T	98% 7,500 T	90% 7,050 T	85% 7,210 T
1206 F	135	"	"	110% 8,150 T	110% 8,150 T	100% 8,500 T	110% 7,680 T	110% 8,000 T	108% 8,050 T	110% 8,000 T
1207 F	131	"	"	108% 8,800 T	108% 8,600 T	100% 8,000 T	110% 7,230 T	100% 7,500 T	108% 8,750 T	110% 8,950 T
1208 F	108	"	"	108% 8,400 T	108% 8,150 T	80% 5,400 T	40% 1,830 T	18% 1,500 T	25% 1,200 T	45% 2,350 T
1209 M	115	"	"	110% 7,500 T	110% 7,500 T	80% 7,000 T	100% 7,230 T	20% 2,500 T	20% 1,750 T	35% 2,100 T
1210 M	152	"	"	106% 7,800 T	106% 7,700 T	60% 4,000 T	33% 1,300 T	25% 1,500 T	20% 1,150 T	20% 1,000 T
1211 M	136	None	Control splenec.	109% 9,000 T	109% 9,700 T	80% 4,500 T	40% 2,060 T	22% 1,200 T	Dead	
1212 M	152	"	"	105% 9,800 T	110% 9,650 T	90% 7,000 T	40% 1,850 T	35% 1,500 T	Dead	
1213 M	141	"	"	105% 9,800 T	100% 9,700 T	70% 7,500 T	20% 1,050 T	20% 1,100 T	10% 960 T	Dead
1214 M	136	Control normal		110% 8,000 T	110% 7,600 T	90% 7,500 T	108% 7,550 T	115% 8,000 T	105% 8,600 T	115% 9,850 T

The letter "T" is used in place of the last three zeros in the red cell count. The hem

* In all the transplants a connective tissue capsule separates the transplant from the su

			Autopsy findings†	Histology of the transplants
17	20	25		
100% 8,200 T	110% 8,600 T	95% 8,600 T	No evidence of anemia Small transplants	* Splenic elements are all present. Follicles are distinct. Pulp cells are regenerated and have large amounts of hemosiderin pigment in their protoplasm. Sinuses contain blood elements. The usual giant cells are present
92% 8,900 T	95% 8,800 T	110% 8,400 T	Two transplants, very large, two resorbed	All elements regenerated and hypertrophied. Follicles are prominent. Pulp cells show hemosiderin pigment. Both endothelial and reticular elements are prominent
100% 9,100 T	90% 8,500 T	110% 8,200 T	Two transplants found, 3 to 4 mm. each	Small transplants. Connecting tissue septum filled with hemosiderin pigment. Follicles small. Pulp cells regenerated and filled with pigment. Central arteries are small
90% 9,350 T	110% 9,100 T	90% 9,200 T	Two transplants, 3 to 4 mm. Two large transplants, 1 cm. in diameter	Small transplants but all elements regenerated. Hemosiderin pigment in the pulp cells. Giant cell occasionally seen as in adult rat spleen. Arterioles small. Reticular and endothelial cells large and prominent
80% 5,200 T	85% 5,250 T	80% 6,200 T	Two transplants, large, 1 cm. and 1.5 cm. Two absorbed	Malpighian corpuscles stand out prominently. Vessels small. Pulp consists of large masses of blood, filling irregular sinuses lined with flat endothelium. There are only scattered reticular and endothelial elements resembling normal spleen. The pulp cells are strikingly absent
80% 5,850 T	85% 6,300 T	100% 7,500 T	Three large transplants, 1.5 cm. in diameter	Transplant similar to 1208. Malpighian corpuscles are prominent. Pulp tissue replaced by red blood cells. Endothelial elements are few and show no pigment
90% 4,200 T	100% 3,750 T	80% 5,100 T	Four large transplants, 1.5, 1.4, 0.8 and 0.8 cm. in diameter	Large transplants. Striking contrast between regenerated follicles and the blood pulp tissue. The sinuses are distended with blood. Endothelium is thin. Reticular cells very sparse
98% 9,400 T	100% 8,600 T	95% 9,100 T		Control normal

expressed in percentages as calculated by the Dare hemoglobinometer.

scale. † All rats killed 74 days after transplantation.

TABLE III
The Effects of Removal of Varying Quantities of Splenic Tissue

No. and sex	Weight gm.	Amount of spleen removed	Blood count before operation	Counts after operation—number of days									
				3	4	7	8	11	13	16	21	27	42
1376 M	225	$\frac{1}{2}$	100% 9,000 T	105% 8,500 T	95% 8,350 T	98% 8,100 T	100% 8,400 T	110% 8,200 T	8,500 T	8,600 T	90% 8,050 T		
1377 M	210	"	88% 7,700 T	95% 7,000 T	100% 7,500 T	110% 7,550 T	110% 7,600 T	115% 8,400 T	7,700 T	8,100 T	100% 8,000 T		
1378 M	250	"	90% 8,500 T	95% 8,750 T	100% 9,500 T	100% 7,200 T	115% 7,200 T	112% 8,500 T	8,700 T	8,500 T	110% 9,200 T		
1380 M	230	$\frac{3}{4}$	105% 9,200 T	100% 9,000 T	110% 8,950 T	95% 8,500 T	100% 8,700 T	115% 9,400 T	9,000 T	9,400 T	105% 9,400 T		
1381 M	252	"	110% 9,250 T	100% 8,900 T	105% 8,900 T	110% 8,600 T	100% 8,800 T	115% 8,500 T	8,900 T	8,500 T	9,000 T		
1382 M	210	"	103% 9,700 T	103% 9,200 T	100% 8,750 T	110% 8,400 T	100% 8,700 T	115% 9,200 T	9,000 T	8,700 T	9,400 T		
1072 M	243	Entire	100% 8,200 T	5,200 T	890 T	Died							
1073 M	267	"	105% 7,500 T	5,490 T	2,030 T	1,460 T		1,960 T	2,000 T	3,820 T	4,500 T	80% 7,300 T	78% 8,450 T
1074 M	252	"	100% 7,900 T	5,400 T	3,230 T	1,730 T		2,080 T	2,900 T	2,790 T	4,200 T	60% 5,250 T	70% 7,000 T

The letter "T" is used in place of the last three zeros in the red cell count. The hemoglobin is expressed in percentages as calculated from readings with the Dare hemoglobinometer.

The peritoneal cavity was exposed by an incision in the upper left quadrant of the abdominal wall, the spleen was gently drawn out of the cavity, a ligature inserted through the mesentery and tied about that portion of the mesentery corresponding to the part of the spleen that was removed. Where the entire spleen was removed a single ligature was tied close to the hilum about the mesentery. The bleeding was slight. The splenic stump was gently replaced in the cavity, care being taken not to tug on the mesentery, the peritoneum and the abdominal wall sutured by continuous sutures. Red blood cell counts before the operation and at frequent intervals after the operation were made.

From Table III it is evident that a small amount of splenic tissue left *in situ* is sufficient to protect the rat against the *Bartonella muris* anemia. This finding is in agreement with Vedder who observed that less than half the spleen will protect against the anemia.

DISCUSSION

A comparative study of the splenic transplants in rats and rabbits indicates a striking difference in the regenerative capacity of the transplants. In the rabbit, Marine and Manley (6) showed that though splenic autotransplants will grow in the adult rabbit in the absence of the spleen in many instances, these transplants are often resorbed or remain small and do not undergo hypertrophy. In the rat, however, we found that splenic autotransplants will grow even in the presence of the spleen and in its absence will undergo marked hypertrophy, growing to the size of accessory spleens or larger. This indicates a difference in the function of the adult spleen of the rat and rabbit.

The observations reported in this paper tend to indicate that the pulp cells are the responsible factor for the specific protective mechanism of the spleen in the rat against the *Bartonella muris* anemia. We have an accurate gauge of physiological activity of the pulp cells of the spleen.

It has been shown in a previous communication (7) that *Trypanosoma lewisi* infection in the adult normal albino rat is accompanied at the height of the infection by a moderate anemia and the appearance in the red blood cells of *Bartonella muris* bodies. Studies on the pathology of this infection indicate that the most significant changes occur in the spleen. At the height of the infection the spleen may enlarge six to ten times. The follicles increase in size and the pulp

cells become engorged with engulfed red blood corpuscles. It is probable that this process of phagocytosis impairs or destroys the normal physiological activity of this group of cells and produces the same effect on the resistance of the organism to the *Bartonella muris* anemia as splenectomy. These observations indicate a specific function of the reticular cells of the spleen.

SUMMARY

Autoplastic splenic transplants were made in adult albino rats 4 weeks and 7 weeks prior to splenectomy and the protective effects against infection with the *Bartonella muris* anemia observed.

1. One-fourth of the spleen left *in situ* will protect adult albino rats against the *Bartonella muris* anemia.

2. Autotransplantation of splenic tissue in adult rats is successful in over 90 per cent of instances.

3. Autoplastic splenic transplants performed 7 weeks prior to splenectomy afford protection against *Bartonella muris* anemia in more than 50 per cent of instances, whereas 4 week old transplants do not protect.

4. A comparative histological study of the transplants of protected and unprotected rats reveals a regeneration of the pulp cells in the protected rats and an exhaustion destruction of the pulp in the unprotected rats.

5. The reticular cells play a specific rôle in protecting the adult albino rat against *Bartonella muris* anemia.

We wish to thank Dr. David Marine for his helpful advice throughout the course of this work.

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EXPLANATION OF PLATE 6

FIG. 1. Rat 1208. Autoplastic splenic transplant performed 7 weeks prior to splenectomy. $M \times 30$. This transplant did not afford protection against *Bartonella* anemia following removal of the spleen. The follicles stand out prominently against the blood filled pulp tissue.

FIG. 2. Rat 1205. Autoplastic splenic transplant performed 7 weeks prior to splenectomy. $M \times 30$. This transplant protected the rat against the *Bartonella muris* anemia following the removal of the spleen. Note the cellular appearance of the pulp.

FIG. 3. Rat 1208. Autoplastic splenic transplant. $M \times 240$ (higher magnification of Fig. 1). Shows exhaustion degeneration of the pulp cells and complete regeneration of follicle.

FIG. 4. Rat 1205. Autoplastic splenic transplant. $M \times 240$ (higher magnification of Fig. 2). Complete regeneration of pulp tissue.





STUDIES IN THE BLOOD CYTOLOGY OF THE RABBIT

IV. CONSECUTIVE NEUTROPHILE, BASOPHILE, AND EOSINOPHILE OBSERVATIONS ON GROUPS OF NORMAL RABBITS

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CORRECTION

On page 6, Vol. 52, No. 1, July 1, 1930, in the second explanatory note at the foot of Table I, the plus sign should be a minus sign; *i.e.*, for + = negative reaction read - = negative reaction.

the individual animal standpoint and the findings have been analyzed upon the basis of consecutive mean values obtained at weekly intervals.

Materials and Methods

These subjects have been fully discussed in connection with the erythrocyte and hemoglobin results (1) and need only be referred to here. The first four groups of rabbits comprised 10 animals each and the fifth group, 5 animals. The period of observation extended from October, 1927 to July, 1929 and the following numbers of weekly examinations were made: Group I, 35; Group II, 13; Group III, 8; Group IV, 29; Group V, 26. For the differential white cell counts, the supravital neutral red technic was used and 100 cells were counted in each specimen.

RESULTS

The results of these experiments dealing with the neutrophile, basophile, and eosinophile cell content of the peripheral blood of normal

TABLE I
Group I—10 Rabbits. Consecutive Values for Neutrophiles, Basophiles, and Eosinophiles

Date	Neutrophiles			Basophiles			Eosinophiles		
	Mean values	Standard deviation	Coefficient of variation	Mean values	Standard deviation	Coefficient of variation	Mean values	Standard deviation	Coefficient of variation
	per cmm.	per cmm.	per cent	per cmm.	per cmm.	per cent	per cmm.	per cmm.	per cent
1927-28									
Oct. 24*	4038 ± 279	1307	32.37	1276 ± 156	730	57.21	273 ± 43	201	73.63
Nov. 1.	4340 ± 295	1383	31.87	791 ± 48	225	28.45	162 ± 12	56	34.57
Nov. 8**	3775 ± 256	1198	31.74	902 ± 85	398	44.12	271 ± 28	131	48.34
Nov. 15.	3266 ± 173	811	24.83	1235 ± 128	602	48.74	400 ± 80	376	94.00
Nov. 22.	5001 ± 388	1820	36.39	1045 ± 122	574	54.93	476 ± 87	408	85.71
Nov. 29.	4470 ± 638	2992	66.94	813 ± 112	525	64.58	211 ± 31	143	67.77
Dec. 6.	3877 ± 394	1848	47.67	842 ± 75	350	41.57	268 ± 29	135	50.37
Dec. 13.	3774 ± 355	1663	44.06	774 ± 95	447	57.75	234 ± 40	185	79.06
Dec. 20.	4905 ± 413	1934	39.43	1024 ± 99	465	45.41	322 ± 32	149	46.27
Dec. 27.	4818 ± 265	1243	25.80	963 ± 124	579	60.12	202 ± 33	154	76.24
Jan. 3.	4618 ± 253	1185	25.66	905 ± 72	336	37.13	288 ± 44	207	71.88
Jan. 10.	4488 ± 365	1712	38.15	1138 ± 129	606	53.25	201 ± 30	142	70.65
Jan. 17.	4566 ± 459	2151	47.11	1180 ± 161	755	63.98	154 ± 30	140	90.91
Jan. 24.	4242 ± 294	1380	32.53	1373 ± 168	787	57.32	277 ± 52	244	88.09
Jan. 31.	5725 ± 529	2481	43.34	6086 ± 157	738	107.58	180 ± 28	130	72.22
Feb. 7.	4820 ± 472	2215	45.95	1358 ± 177	831	61.19	299 ± 40	189	63.21
Feb. 14.	5688 ± 530	2485	43.69	763 ± 65	305	39.97	193 ± 37	173	89.64
Feb. 21.	4544 ± 366	1715	37.74	1710 ± 113	529	30.94	290 ± 42	197	67.93
Feb. 28.	6687 ± 883	4142	61.94	1111 ± 157	735	66.16	244 ± 46	215	88.11
Mar. 6.	4790 ± 289	1356	28.31	1291 ± 233	1092	84.59	308 ± 45	210	68.18
Mar. 13.	5208 ± 459	2151	41.30	787 ± 94	443	56.29	191 ± 40	186	97.38
Mar. 20.	6653 ± 816	3826	57.51	707 ± 75	353	49.93	255 ± 65	305	119.61

Mar. 27.....	4662 ± 375	1756	37.67	1231 ± 115	538	43.70	170 ± 33	153	90.00
Apr. 3.....	4851 ± 367	1719	35.44	1046 ± 108	506	48.37	306 ± 33	154	50.33
Apr. 10.....	5793 ± 401	1880	32.45	1078 ± 127	597	55.38	400 ± 58	274	68.50
Apr. 17.....	5060 ± 359	1684	33.28	1108 ± 124	579	52.26	217 ± 23	109	50.23
Apr. 24.....	5256 ± 516	2419	46.02	1046 ± 92	429	41.01	220 ± 57	268	121.82
May 1.....	4851 ± 278	1304	26.88	1121 ± 69	321	28.64	159 ± 39	183	115.09
May 8.....	5055 ± 401	1880	37.19	1399 ± 173	813	58.11	190 ± 30	139	73.16
May 15.....	5111 ± 382	1791	35.04	1234 ± 107	501	40.60	416 ± 89	416	85.58
May 22.....	5901 ± 597	2797	47.40	1103 ± 130	609	55.21	213 ± 33	155	72.77
May 29.....	5269 ± 382	1789	33.95	981 ± 83	389	39.65	193 ± 29	135	69.95
June 5.....	5751 ± 361	1691	29.40	1462 ± 127	595	40.70	233 ± 50	232	99.57
June 12.....	5048 ± 334	1567	31.04	1339 ± 162	760	56.76	270 ± 28	133	49.26
June 19.....	5343 ± 425	1992	37.28	1364 ± 183	857	62.83	268 ± 42	197	73.51
Mean.....	4919 ± 268	2349	47.75	1091 ± 27	238	21.81	256 ± 9	76	29.69
Minimum.....	3266			686			154		
Maximum.....	6687			1710			476		

* October 24 and 26.

** November 4 and 9.

TABLE II
Group II—10 Rabbits. Consecutive Values for Neutrophiles, Basophiles, and Eosinophiles

Date	Neutrophiles			Basophiles			Eosinophiles		
	Mean values	Standard deviation	Coefficient of variation	Mean values	Standard deviation	Coefficient of variation	Mean values	Standard deviation	Coefficient of variation
	per cmm.	per cmm.	per cent	per cmm.	per cmm.	per cent	per cmm.	per cmm.	per cent
1928									
Mar. 29.....	3582 ± 284	1330	37.13	579 ± 42	197	34.02	139 ± 25	116	83.45
Apr. 3.....	3821 ± 220	1031	26.98	768 ± 88	411	53.52	155 ± 19	91	58.71
Apr. 10.....	3825 ± 340	1593	41.65	550 ± 55	258	46.91	140 ± 25	117	83.57
Apr. 17.....	4481 ± 320	1501	33.50	882 ± 94	442	50.11	277 ± 76	358	129.24
Apr. 24.....	4420 ± 403	1890	42.76	859 ± 110	520	60.54	152 ± 18	86	56.58
May 1.....	3123 ± 106	497	15.91	831 ± 108	505	60.77	150 ± 26	124	82.67
May 8.....	4111 ± 280	1311	31.89	871 ± 49	231	26.52	111 ± 21	96	86.49
May 15.....	3515 ± 359	1685	47.94	590 ± 65	306	51.86	180 ± 27	128	71.11
May 22.....	4920 ± 333	1559	31.69	954 ± 100	467	48.95	258 ± 33	155	60.08
May 29.....	4008 ± 350	1642	40.97	726 ± 88	411	56.61	113 ± 30	141	124.78
June 5.....	4348 ± 281	1319	30.34	1103 ± 84	392	35.54	118 ± 18	86	72.88
June 12.....	3653 ± 213	999	27.35	988 ± 113	528	55.11	111 ± 16	76	68.47
June 19.....	4632 ± 190	889	19.19	856 ± 109	510	59.58	175 ± 25	116	66.29
Mean.....	4034 ± 92	491	12.17	810 ± 29	157	19.38	160 ± 10	51	31.88
Minimum.....	3123			550			111		
Maximum.....	4920			1103			277		

TABLE III
Group III—10 Rabbits. Consecutive Values for Neutrophiles, Basophiles, and Eosinophiles

Date	Neutrophiles			Basophiles			Eosinophiles		
	Mean values	Standard deviation	Coefficient of variation	Mean values	Standard deviation	Coefficient of variation	Mean values	Standard deviation	Coefficient of variation
	per cmm.	per cmm.	per cent	per cmm.	per cmm.	per cent	per cmm.	per cmm.	per cent
1928									
Sept. 20.....	4777 \pm 484	2270	47.52	437 \pm 42	198	45.31	144 \pm 47	221	153.47
Sept. 28.....	3002 \pm 163	762	25.13	586 \pm 50	232	39.59	179 \pm 30	140	78.21
Oct. 10.....	3913 \pm 467	2188	55.93	671 \pm 84	395	58.87	215 \pm 32	149	69.30
Oct. 19.....	2682 \pm 263	1235	46.05	835 \pm 66	308	36.89	157 \pm 18	86	54.78
Nov. 2.....	3853 \pm 281	1315	34.13	733 \pm 64	301	41.06	168 \pm 39	184	109.52
Nov. 9.....	3620 \pm 213	1000	27.16	836 \pm 102	477	57.06	257 \pm 47	218	84.82
Nov. 16.....	3004 \pm 261	1223	40.71	684 \pm 57	267	39.04	252 \pm 34	157	62.30
Nov. 22.....	3006 \pm 249	1167	38.44	708 \pm 69	321	45.34	178 \pm 25	115	64.61
Mean.....	3482 \pm 154	647	18.58	686 \pm 29	122	17.78	194 \pm 10	40	20.62
Minimum.....	2682			437			144		
Maximum.....	4777			836			257		

TABLE IV
Group IV—10 Rabbits. Consecutive Values for Neutrophils, Basophiles, and Eosinophiles

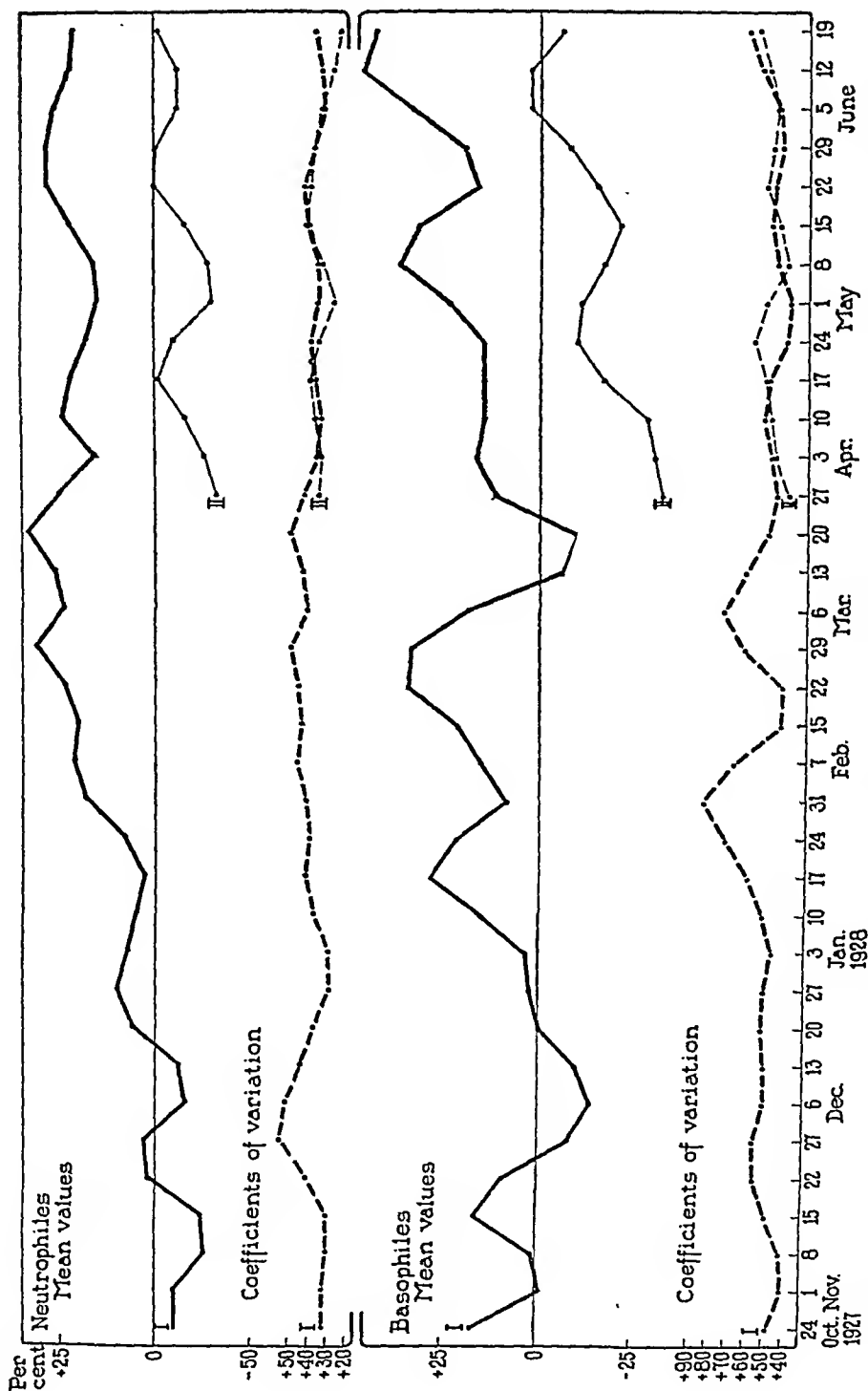
Date	Neutrophiles			Basophiles			Eosinophiles		
	Mean values	Standard deviation	Coefficient of variation	Mean values	Standard deviation	Coefficient of variation	Mean values	Standard deviation	Coefficient of variation
	per cmm.	per cmm.	per cent	per cmm.	per cmm.	per cent	per cmm.	per cmm.	per cent
1928-29									
Nov. 27.	3880 \pm 246	1154	29.75	602 \pm 86	401	66.67	111 \pm 25	118	106.62
Dec. 4.	3639 \pm 286	1342	36.88	742 \pm 96	450	60.60	73 \pm 14	64	88.39
Dec. 11.	3535 \pm 280	1312	37.11	812 \pm 55	257	31.59	120 \pm 20	96	80.06
Dec. 18.	2938 \pm 158	742	25.23	668 \pm 77	360	53.87	64 \pm 14	64	99.56
Dec. 26.	3440 \pm 258	1208	35.12	670 \pm 74	349	52.15	82 \pm 18	86	104.89
Jan. 2.	3530 \pm 174	816	23.12	735 \pm 63	297	40.39	55 \pm 15	71	128.90
Jan. 8.	2870 \pm 279	1310	45.64	608 \pm 63	296	48.61	117 \pm 28	133	113.73
Jan. 15.	3396 \pm 172	808	23.78	573 \pm 64	300	52.31	117 \pm 17	81	69.22
Jan. 22.	3221 \pm 255	1195	37.11	758 \pm 77	362	47.81	123 \pm 21	100	81.44
Jan. 29.	4442 \pm 292	1371	30.87	965 \pm 83	387	40.14	103 \pm 20	95	92.19
Feb. 5.	3481 \pm 331	1551	44.55	552 \pm 36	167	30.16	83 \pm 18	84	100.69
Feb. 13.	5170 \pm 378	1771	34.27	632 \pm 78	367	58.03	177 \pm 29	137	77.51
Feb. 19.	6085 \pm 328	1536	25.24	741 \pm 71	334	45.02	224 \pm 61	285	127.40
Feb. 26.	5403 \pm 360	1687	31.21	850 \pm 127	594	69.87	112 \pm 22	101	90.06
Mar. 12.	4251 \pm 215	1010	23.75	408 \pm 53	247	60.53	72 \pm 16	77	107.43
Mar. 19.	5544 \pm 426	1996	36.00	514 \pm 30	142	27.52	201 \pm 30	139	69.35
Mar. 26.	5483 \pm 351	1644	29.97	400 \pm 44	204	51.00	44 \pm 10	47	105.90
Apr. 2.	5342 \pm 384	1799	33.67	612 \pm 58	273	44.53	105 \pm 25	115	109.60
Apr. 9.	5160 \pm 133	625	12.11	377 \pm 31	147	39.08	149 \pm 26	124	83.08
Apr. 16.	5396 \pm 314	1470	27.24	434 \pm 56	262	60.36	55 \pm 16	76	137.40
Apr. 23.	5609 \pm 218	1023	18.23	505 \pm 57	267	52.90	132 \pm 39	185	140.10
Apr. 30.	5384 \pm 220	1031	19.14	474 \pm 66	311	65.54	54 \pm 15	70	128.90

May 7.....	5434 ± 332	1557	28.66	479 ± 60	280	58.41	153 ± 28	130	84.64
May 14.....	4380 ± 199	883	20.15	490 ± 104	464	94.59	106 ± 8	35	117.63
May 21.....	4826 ± 248	1101	22.80	388 ± 75	332	85.47	26 ± 12	55	210.00
May 28.....	4769 ± 231	1026	21.51	564 ± 100	443	78.58	87 ± 27	122	139.65
June 4.....	4123 ± 263	1170	28.37	386 ± 52	231	59.85	36 ± 13	56	155.27
June 11.....	4085 ± 209	930	22.77	455 ± 68	301	66.04	37 ± 9	42	114.59
June 18.....	3464 ± 164	688	19.86	343 ± 50	211	61.57	76 ± 17	71	93.00
Mean.....	4423 ± 117	933	21.09	577 ± 20	157	27.20	100 ± 6	48	48.01
Minimum.....	2870			343			26		
Maximum.....	6085			965			224		

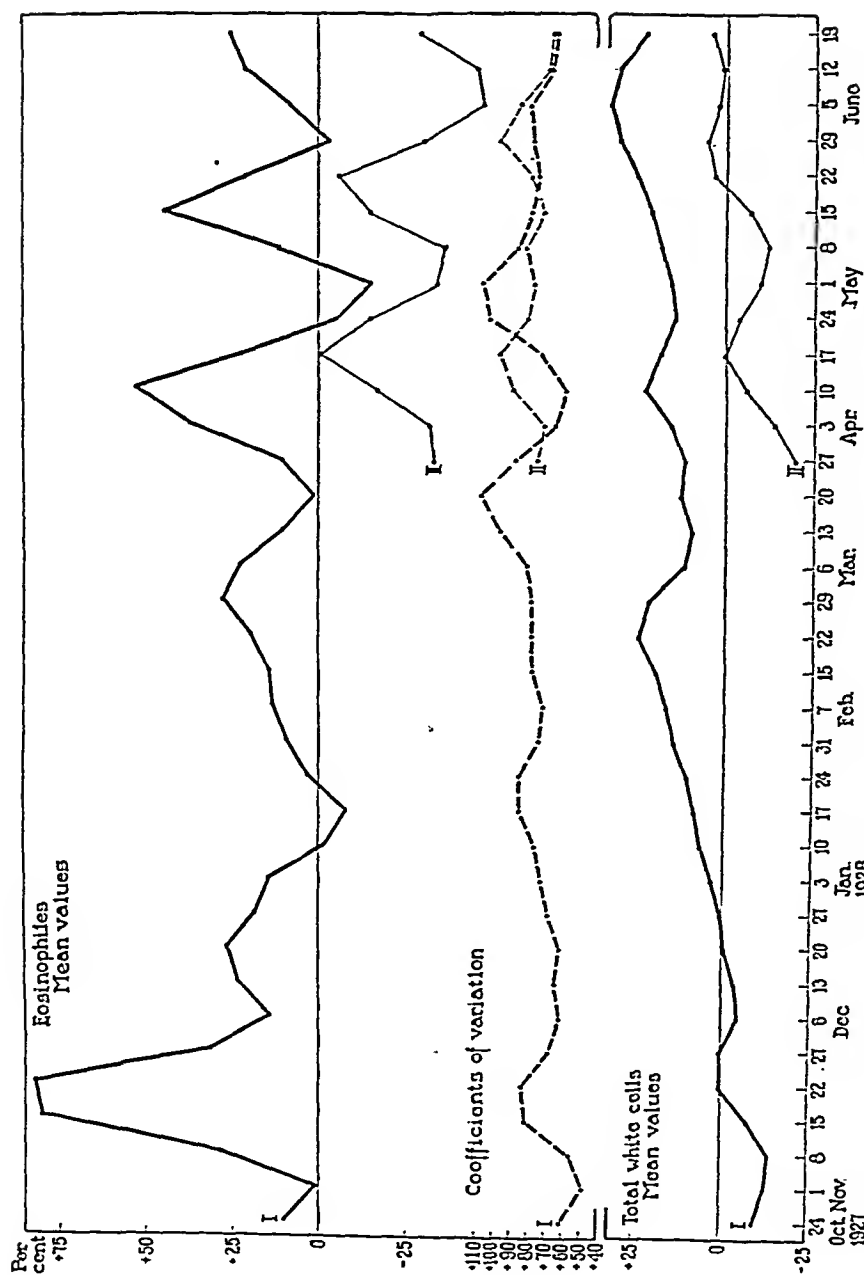
TABLE V
Group V—5 Rabbits. Consecutive Values for Neutrophiles, Basophiles, and Eosinophiles

Date	Neutrophiles			Basophiles			Eosinophiles		
	Mean values	Standard deviation	Coefficient of variation	Mean values	Standard deviation	Coefficient of variation	Mean values	Standard deviation	Coefficient of variation
	per cmm.	per cmm.	per cent	per cmm.	per cmm.	per cent	per cmm.	per cmm.	per cent
1928-29									
Dec. 21.....	2062 ± 191	632	30.65	290 ± 57	188	64.83	17 ± 6	21	123.53
Dec. 31.....	1892 ± 197	654	34.57	498 ± 66	220	44.18	19 ± 7	24	126.32
Jan. 3.....	2815 ± 326	1081	38.40	614 ± 41	135	21.99	117 ± 32	106	90.60
Jan. 10.....	1915 ± 251	833	43.50	525 ± 46	151	28.76	74 ± 15	51	68.92
Jan. 24.....	2014 ± 115	382	18.97	381 ± 38	127	33.33	36 ± 13	44	122.22
Jan. 31.....	2097 ± 272	902	43.01	642 ± 171	568	88.47	73 ± 13	42	57.53
Feb. 7.....	1994 ± 162	537	26.97	333 ± 59	196	58.86	124 ± 39	130	104.84
Feb. 15.....	2999 ± 392	1298	43.28	529 ± 92	304	57.47	121 ± 26	85	70.25
Feb. 21.....	3677 ± 176	584	15.88	461 ± 29	96	20.82	156 ± 35	117	75.00
Mar. 1.....	2850 ± 84	279	9.79	507 ± 88	292	57.59	190 ± 39	130	68.42
Mar. 8.....	3320 ± 145	480	14.46	454 ± 20	65	14.32	147 ± 21	68	46.26
Mar. 15.....	2680 ± 135	449	16.75	656 ± 46	224	34.15	226 ± 53	175	77.43
Mar. 22.....	3012 ± 140	465	15.44	363 ± 61	202	55.65	219 ± 38	127	57.99
Mar. 29.....	3650 ± 283	937	25.67	484 ± 118	390	80.58	189 ± 41	136	71.96
Apr. 5.....	3669 ± 207	685	18.67	512 ± 43	144	28.13	180 ± 20	67	37.22
Apr. 12.....	3094 ± 186	617	19.94	382 ± 62	204	53.40	181 ± 35	115	63.54
Apr. 19.....	4171 ± 246	817	19.59	410 ± 32	106	25.85	153 ± 36	118	77.12
Apr. 26.....	3715 ± 213	705	18.98	528 ± 76	253	47.92	98 ± 24	80	81.63
May 3.....	3612 ± 294	975	26.99	615 ± 57	190	30.89	127 ± 39	130	102.36
May 10.....	3171 ± 240	797	25.13	458 ± 64	211	46.07	208 ± 67	221	106.25
May 17.....	3774 ± 322	1068	28.30	461 ± 40	132	28.63	115 ± 29	95	82.61

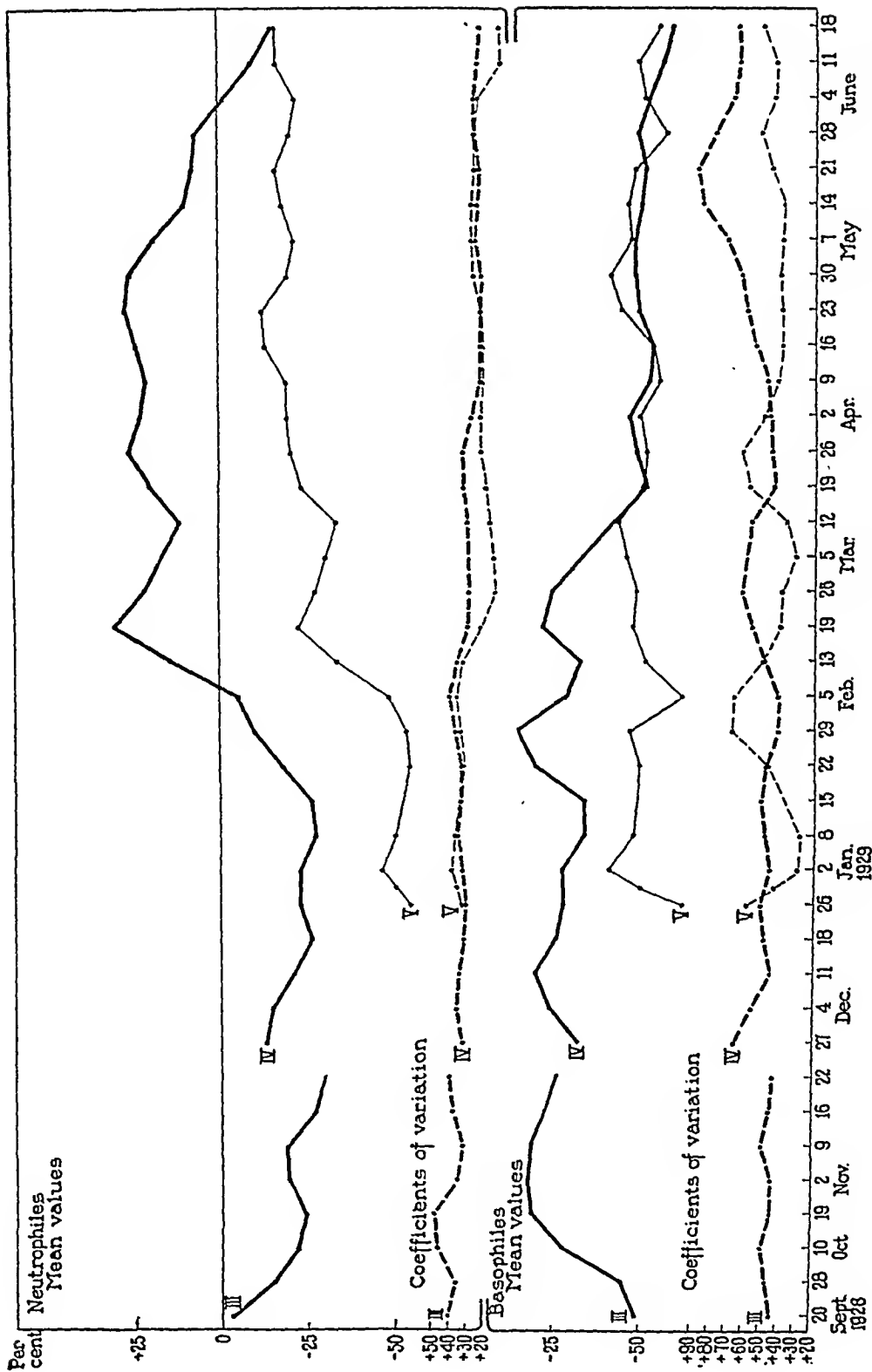
May 24.....	3604 ± 239	791	21.95	634 ± 86	284	44.79	126 ± 16	54	42.86
May 31.....	3616 ± 276	916	25.33	212 ± 36	120	56.60	37 ± 14	47	12.70
June 7.....	3045 ± 215	714	23.45	546 ± 66	220	40.29	126 ± 35	115	91.27
June 14.....	4005 ± 227	752	18.78	492 ± 54	178	36.18	127 ± 41	135	106.30
June 21.....	3519 ± 211	698	19.84	382 ± 65	214	56.02	194 ± 50	164	84.54
Mean.....	3076 ± 92	692	22.50	476 ± 14	109	22.90	130 ± 8	59	45.38
Minimum.....	1892			212			17		
Maximum.....	4171			652			226		



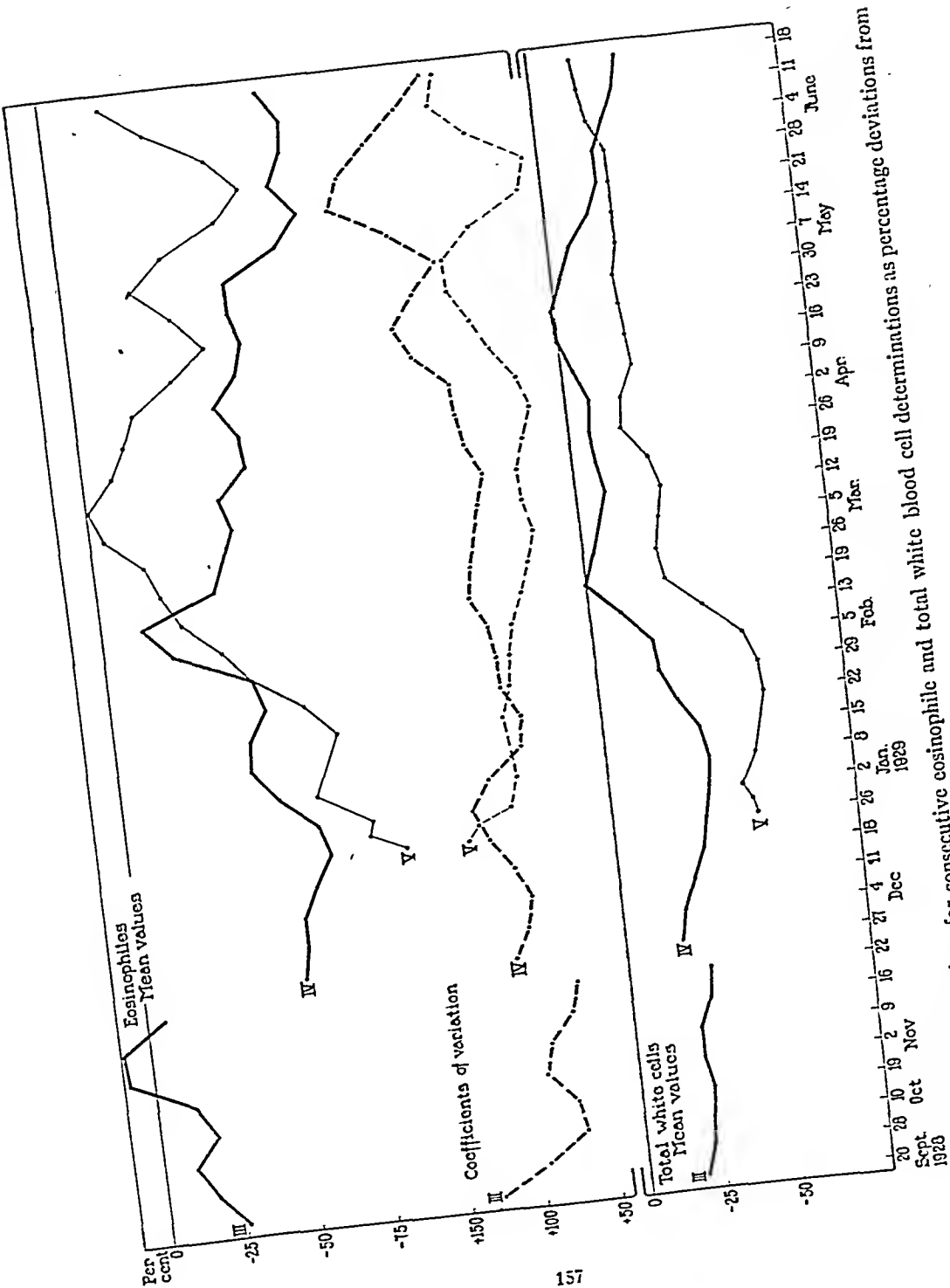
TEXT-FIG. 1. Mean values for consecutive neutrophile and basophile determinations as percentage deviations from standard values. 1927-28.



TEXT-FIG. 2. Mean values for consecutive eosinophile and total white blood cell determinations as percentage deviations from standard values, 1927-28.



TEXT-FIG. 3. Mean values for consecutive neutrophile and basophile determinations as percentage deviations from standard values, 1928-29.



TEXT-FIG. 4. Mean values for consecutive eosinophilic and total white blood cell determinations as percentage deviations from standard values, 1928-29.

rabbits examined over long periods of time are presented in the form of consecutive weekly mean values for each group of animals (Tables I to V). In addition, the probable errors of the means, the standard deviations, and the coefficients of variation are given. The series of curves in Text-figs. 1 to 4 representing the mean values, are drawn from the percentage deviations of the smoothed means from the following standard values (1, 4): neutrophiles, 4340 cells per cubic millimeter; basophiles, 950 cells per cubic millimeter; eosinophiles, 215 cells per cubic millimeter. The curves of the smoothed coefficients of variation of the means are also given in Text-figs. 1 to 4. These charts also include curves which represent the total white cell means in terms of the percentage deviations of the smoothed means from a standard value of 9560 cells per cubic millimeter, in order that comparisons may readily be made between the results on the various granular leucocytes and those on the total white count without referring to the preceding paper (2) in which the latter observations are discussed.

DISCUSSION AND SUMMARY

For convenience of discussion, the results of these experiments dealing with consecutive neutrophile, basophile, and eosinophile counts will be considered in the chronological order of the five groups of rabbits studied.

Group I was examined from October 24, 1927 to June 21, 1928 (Table I). It will be seen by referring to Text-fig. 1, that the mean numbers of neutrophiles had increased approximately 30 per cent by the second half of the experiment and that thereafter, this higher level was generally maintained. In October, November, and December, the general level of the curve fluctuates slightly below and above the standard value of 4340 cells per cubic millimeter represented by the base line. During January, February, and March, a decided upward trend is noted and thereafter to the last of June, a slightly lower level is observed.

The mean numbers of basophiles in Group I (Table I) were also increased during the experiment, but as will be seen from the curve in Text-fig. 1, the change was accomplished in a much more irregular fashion than in the case of the neutrophiles. During October and November, the general trend of the curve is downward from an

initial level above the base line (950 cells per cubic millimeter); in December, January, and February, the curve describes an irregular but marked rise which is followed by an abrupt fall to the November level; at the end of March and continuing through April, May, and June, there is a second rise which carries the curve to the highest level observed (55 per cent above the base line). During these periods of increasing value, there were two interruptions, the first occurring the end of January and the second in May.

In the case of the eosinophiles of Group I (Table I) pronounced variations in mean numbers were observed, as is shown by the curve in Text-fig. 2. Throughout November and again in April and May, large numbers of these cells were observed which account for the three prominent and abrupt peaks of the curve. Following each marked increase, the mean values sank to a comparatively low level, but on the whole, the means throughout the experiment were higher than the standard value of 215 cells per cubic millimeter.

Comparing the general changes in the mean values of the three classes of granular leucocytes with that of the total white cells (Text-fig. 2), it will be noted that during the first half of the experiment (October to March) increasing values were observed in the total white cells, the neutrophiles, and the basophiles (after November) which are indicated by the upward direction of their curves. On the other hand, this feature was not found in the eosinophile results until the January and February observations. During the latter part of February and throughout March, lower levels prevailed in the total white cell, the basophile, and the eosinophile means but in the case of the neutrophiles, the values although comparatively irregular, were on the whole not diminished. In the last period of the experiment, the trend of the total white cells was again upward, a movement characteristic also of the basophiles and to a slight extent of the neutrophiles. The later eosinophile means which showed no consistent trend in one direction or another were characterized by a series of abrupt and pronounced swings. The high points of these fluctuations (February 29, April 10, and May 15), however, occurred at the time of high neutrophile values and somewhat analogous findings were observed in the earlier weeks of the experiment (November 22 and December 20).

The coefficients of variation of the neutrophile means of Group I (Table I) are fairly uniform although their order of magnitude is high.

The curve of the smoothed values (Text-fig. 1) shows variations within a 10 per cent range at the general level of 30 to 40 per cent except in November and December when the abrupt alteration of mean values is accompanied by a sharp upward turn of the coefficient curve. In the case of the basophiles (Table I) the curve of the smoothed coefficients of variation (Text-fig. 1) is fairly regular at the 40 to 50 per cent level except for two major upward swings the end of January and the first of March which accompany not increased, but diminished mean values. In the last third of the experiment, during which the basophile means rose to the highest figure observed, the coefficients of variation are comparatively uniform. The coefficients of variation of the eosinophile means (Table I) are of a very high order of magnitude, the curve of the smoothed values ranging from 47 to 107 per cent. The highest points on the curve correspond to the period of extremely high mean values in November but to the low mean values in January, March, and May which immediately succeeded abruptly increased counts. During these phases of augmented mean values, the coefficients of variation are smaller so that the curve is directed downward.

Group II was examined from March 29 to June 19, 1928 (Table II). The curves illustrating the neutrophile, the basophile, and the eosinophile means as percentage deviations from standard values (Text-figs. 1 and 2) show a striking resemblance in general contour to the curves of Group I for this period as is also the case with the total white cells (Text-fig. 2); the levels of the means of Group II, however, are considerably lower. The two prominent peaks of the eosinophile curve in April and May, occurring almost at the same time as those of Group I, are worthy of special note in view of the comparatively small numbers of these cells.

The coefficients of variation of these three cell means of Group II (Table II) are of the same order of magnitude as those of Group I. The curves of the smoothed values (Text-figs. 1 and 2) are similar to those of Group I except in the case of the eosinophiles in which one of the two highest points corresponds to and the other directly follows, high mean values.

Group III was examined from September 20 to November 22, 1928 (Table III). The neutrophile, basophile, and eosinophile mean values were generally lower than those of Group I and were more comparable to those of Group II, as may be seen by comparing the respective curves in Text-figs. 1, 2, 3, and 4. In the case of the neutro-

phile curve, there is a prevailing downward trend which is described in a fairly regular fashion while the direction of the basophile and eosinophile curves is upward. The curve for the total white cells, on the other hand, is almost a straight line (Text-fig. 4).

The coefficients of variation of these means (Table III) are of similar orders of magnitude to those of Groups I and II, but contrary to what was found in Group I, the curve of the smoothed neutrophile values shows more irregularities than that of the basophiles (Text-fig. 3). The highest point of the eosinophile coefficient curve (Text-fig. 4) corresponds to the smallest mean value, a feature which was generally characteristic of Group I.

Group IV was examined from November 27, 1928 to June 18, 1929 (Table IV). On the whole, the mean values for the neutrophile, the basophile, and the eosinophile cells were generally smaller than those of Group I. In the case of the neutrophiles (Text-fig. 3), low initial levels prevailed until the middle of January, at which time a pronounced increase occurred and a level of approximately 25 per cent above the standard value was maintained until the end of April, after which progressively smaller values were found. In general form, the neutrophile curve resembles that of the total white cells. The highest basophile values, on the other hand, were observed in the first half of the experiment as is shown by the curve in Text-fig. 3; in the second half, the curve occupies a uniform position 25 per cent lower than its first level. The two small peaks in the basophile curve at the end of January and toward the end of February occur at the time of the pronounced upward swing of the neutrophile curve. The trend of the first part of the eosinophile curve (Text-fig. 4) is in a rising direction culminating abruptly in a sharp peak the latter part of February. From then onward to the end of the experiment, the curve describes an irregular fall and ends at a lower level than that of the initial observations.

The coefficients of variation of the neutrophile, basophile, and eosinophile means of Group IV (Table IV) are comparable in their orders of magnitude to those of the other groups. From the curves of the smoothed values, it will be seen that that of the neutrophiles was more uniform than that of the basophiles (Text-fig. 3) which is the case with Groups I and II but not with Group III. It should be noted that the marked changes in the level of the neutrophile mean values are not accompanied by pronounced fluctuations of the coefficient

curve. The general level of the basophile coefficient curve is fairly well maintained until April and May when an upward swing occurs although no accompanying change in the curve representing the mean values is seen. In the case of the eosinophiles (Text-fig. 4), the coefficient curve is fairly regular until April and May when there are two abrupt upward fluctuations. These changes occur during diminishing mean values and the second high coefficient peak follows a marked drop in the mean values during the first part of May.

Group V was examined from December 29, 1928 to June 21, 1929 (Table V). The curves representing the mean values of the neutrophile, basophile, and eosinophile cells (Text-figs. 3 and 4) are similar in general outline to those of Group IV examined during the same months. In the case of the neutrophiles, however, much lower values prevailed until the end of the experiment. A comparison of the neutrophile and total white cell curves brings out the fact of their similarity with respect to general form as was also found with the other groups. The basophile curve for Group V which is relatively stable at a level of 50 per cent below the standard value shows the smallest fluctuations of any of the basophile curves. In many places, however, those which do occur correspond to similar alterations in the curve of the Group IV basophiles. In the case of the eosinophiles, the curves of the two groups bear a general resemblance to each other but the swings of the Group V curve are less pronounced, the summit of its marked rise in the middle of the observation period occurs somewhat later, and its second half occupies a higher level.

The coefficients of variation of the neutrophile means of Group V (Table V) are generally comparable to those of Group IV (Text-fig. 3); those of the basophiles (Table V) are on the whole somewhat smaller (Text-fig. 3). The coefficients of the eosinophile means of Group V are generally smaller than those of Group IV (Table IV). In the curve of the smoothed values (Text-fig. 4) it should be noted that comparatively low levels prevail during February and March in which months the mean values were progressively high. In its latter portion, the curve described two abrupt upward swings, the first of which corresponds to decreasing and the second to increasing mean values.

There are certain features of these results obtained from the observations of many weeks to which attention may now be especially directed. In the first place, the neutrophiles, as would be expected in view of the relative number of these cells, influence the total white cell count to the extent that the curve representing the consecutive

smoothed mean values of the one usually resembles quite closely the curve of the other.

In the experiments carried out for the longer periods, Groups I and II in 1927-28 and Groups IV and V in 1928-29, an increase in the neutrophile mean values occurred in the late winter and early spring months but during May and June, lower or stationary levels prevailed. In Group III examined during the autumn of 1928, the trend was in the direction of smaller values, a finding which is in accord with the low values of the other groups in October, November, and December of both years. The results pertaining to the basophiles differed in the two years. In 1927-28, there was a general increase in mean values which continued through the spring months; in the second year, the trend was toward smaller values. The rising basophile values of Group III in the fall of 1928 were in accord with the early findings of Group IV, the first examination of which followed the last observations of Group III. In the case of the eosinophiles, the results were more irregular. Their most prominent feature was the abrupt and very pronounced increase and decrease of mean values occurring in November of 1927 and in the late winter and spring months of 1928 and 1929.

That the variations in the general levels of the neutrophile, the basophile, and the eosinophile mean values observed in these experiments have a statistical significance is shown by the following examples of the ratios of the differences of certain means to their probable errors:

Group number	Neutrophiles			Basophiles			Eosinophiles		
	Dates of mean values		Ratio	Dates of mean values		Ratio	Dates of mean values		Ratio
I	Nov. 15	June 5	6.21	Nov. 1	June 19	3.03	Mar. 13	Apr. 10	2.99
	Nov. 15	Jan. 31	4.48	Jan. 31	Feb. 21	5.28	Apr. 10	May 1	3.44
II	Mar. 29	June 19	3.07	Mar. 29	June 5	5.57	May 8	May 22	3.77
	May 1	May 22	5.15	May 15	June 5	4.84	May 15	June 12	3.97
III	Sept. 20	Oct. 19	3.81	Sept. 20	Oct. 19	5.10	Sept. 20	Nov. 9	1.69
IV	Jan. 2	Feb. 19	6.89	Nov. 27	Jan. 29	3.04	Jan. 2	Feb. 19	2.68
	Apr. 23	June 18	7.89	Jan. 29	Mar. 26	6.73	Feb. 13	June 11	4.67
V	Dec. 29	June 21	5.12	Dec. 29	June 21	2.94	Dec. 29	Mar. 15	3.94
	Jan. 10	Feb. 21	5.76	Jan. 3	Feb. 7	3.90	Mar. 15	Apr. 26	2.21

In general, it was found that in two groups of rabbits examined during the same months, the variations in the mean levels of the three classes of granular leucocytes were similar both as regards the direction of the change and the time at which it occurred. This group resemblance was particularly striking in the case of the marked fluctuations of the eosinophiles but it was also characteristic of the neutrophiles and the basophiles. The results of the two years differed in that the general level of the neutrophile, the basophile, and the eosinophile mean values was higher in the groups examined in 1927-28 than in those observed in 1928-29. Both features of group resemblance and the yearly difference of level were also characteristic of the results pertaining to the red blood cells and the hemoglobin content (1) and the total white, the granular, and the non-granular cells (2). In the case of the total white count and its granular and non-granular components, the period of greatest irregularity of mean values occurred in the late winter and spring months of both years. This was true of the neutrophiles in the second year but to a less extent in the first year. The basophile values were very variable throughout the first year while in the second, the greatest irregularities occurred in the winter months. The fluctuations of the eosinophiles were most pronounced in the late winter and spring months of both years.

In the majority of cases, the physical condition of the rabbits throughout the periods of observation was excellent and gains in body weight were the rule. There were, however, a number of instances of clinical snuffles of slight or moderate degree as well as several cases of ear canker. The effect of these conditions upon the blood count will subsequently be taken up in detail when the results of these and other experiments are considered from the standpoint of the individual animal and of a comparison with snuffles free stock; at present the subject can only be referred to briefly. There were 2 deaths among the 45 rabbits of these experiments, occurring in Group IV; both animals had had snuffles and in one, a chronic nephritis was found at postmortem examination. It appeared that the occurrence of a muco-purulent nasal discharge was most frequently associated with an increase of neutrophiles and in certain instances, of lymphocytes, while in a few cases, higher monocyte values were observed. The persistence of a nasal discharge was not, however,

necessarily accompanied by continued high counts. On the other hand, increased counts were observed in rabbits in which no intercurrent infection could be demonstrated by repeated clinical examination and in addition, comparable fluctuations of cell levels were found in both "normal" rabbits and in those with slight or moderate snuffles. The effect of ear canker upon the blood count was not clearly defined. In certain animals, its occurrence coincided with a transitory increase of the neutrophile and lymphocyte cells, while in others, no characteristic alteration of cell levels was observed. Although these conditions of intercurrent infection undoubtedly had an effect upon the blood count, nevertheless, they do not appear to be a factor of major importance with respect to the character of the general variations in cell levels observed over long periods of time from the standpoint of the consecutive mean values of these animal groups.

CONCLUSIONS

Consecutive weekly observations on the neutrophile, the basophile, and the eosinophile counts of the peripheral blood were made on 5 groups of normal rabbits, a total of 45 animals, during a period of 20 months from October, 1927 to July, 1929. Individual groups were examined 8 to 35 weeks.

In the case of the 4 groups followed 13 to 35 weeks, the general trend of the neutrophile cells was towards increased mean values; with the group followed 8 weeks, decreasing values were found. An increase in the mean values of the basophile cells was observed in the two groups of rabbits followed in 1927-28; in the groups of 1928-29, the mean values decreased. The mean values of the eosinophile cells showed no definite trends but the findings were characterized by abrupt and marked fluctuations.

The periods of greatest irregularity in mean neutrophile and eosinophile values occurred in the fall and the late winter and spring months of both years, but in the case of the basophiles, the irregularities were distributed throughout the first year and occurred chiefly in the winter months of the second year.

The major trends and many of the minor fluctuations as well which were observed in the mean cell values of one group of rabbits were also generally seen in another group examined during the same months.

The general levels of the neutrophile, the basophile, and the eosinophile mean values in the groups examined during 1927-28 were higher than in the groups of 1928-29.

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STUDIES IN THE BLOOD CYTOLOGY OF THE RABBIT

V. CONSECUTIVE LYMPHOCYTE AND MONOCYTE OBSERVATIONS ON GROUPS OF NORMAL RABBITS

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Previous papers of this series have contained the results of repeated blood examinations on 5 groups of normal male rabbits with respect to the erythrocyte and hemoglobin determinations (1), the total white cell contents (2), and the neutrophile (pseudo-eosinophile), the basophile, and the eosinophile cell counts (3). The purpose of these experiments was to obtain information on the general character or trend of the spontaneous variations described by the cellular constituents of the peripheral blood over prolonged periods of time, and the data obtained have, in each instance, been presented on the basis of the consecutive weekly mean values of each group. In the present paper, the results on the non-granular cells—the lymphocytes and the monocytes—are reported.

Materials and Methods

A description of the materials and methods employed in these experiments and of the method of analyzing the results obtained has been given in the paper dealing with the observations on the erythrocytes and hemoglobin (1). Suffice it to say here that the period of time covered by the experiments extended from October, 1927 to July, 1929, and in the majority of cases, the blood was examined at weekly intervals. The numbers of examinations were: Group I, 35; Group II, 13; Group III, 8; Group IV, 29; Group V, 26. Each of the first 4 groups comprised 10 and the last, 5 normal male rabbits. The supravital neutral red technic was used in making the differential white cell counts and 100 cells were counted in each specimen.

RESULTS

The consecutive lymphocyte and monocyte determinations on 5 groups of normal rabbits are given in Tables I to V in the form of mean

TABLE I

Group I—10 Rabbits. Consecutive Values for Lymphocytes and Monocytes

Date	Lymphocytes			Monocytes		
	Mean values	Standard deviation	Coefficient of variation	Mean values	Standard deviation	Coefficient of variation
1927-28	per cmm.	per cmm.	per cent	per cmm.	per cmm.	per cent
Oct. 24*.....	2253 ± 185	867	25.97	987 ± 83	388	39.31
Nov. 1.....	1861 ± 92	431	23.16	1092 ± 139	650	59.52
Nov. 8**.....	1929 ± 165	772	40.02	1153 ± 104	487	42.24
Nov. 15.....	2560 ± 105	491	19.18	1085 ± 83	389	35.85
Nov. 22.....	2005 ± 169	794	39.60	1409 ± 185	869	61.67
Nov. 29.....	2984 ± 210	983	32.94	1209 ± 174	817	67.63
Dec. 6.....	2781 ± 238	1117	40.17	1073 ± 114	533	49.67
Dec. 13.....	3171 ± 399	1871	59.00	1178 ± 127	596	50.59
Dec. 20.....	2209 ± 166	778	35.22	1231 ± 127	595	48.33
Dec. 27.....	2554 ± 197	922	36.10	864 ± 77	362	41.90
Jan. 3.....	2945 ± 333	1559	52.94	1045 ± 52	245	23.44
Jan. 10.....	3425 ± 256	1202	35.09	967 ± 73	341	35.26
Jan. 17.....	3429 ± 352	1648	49.11	1025 ± 102	480	46.83
Jan. 24.....	3511 ± 285	1336	38.05	967 ± 58	270	27.92
Jan. 31.....	3444 ± 284	1332	38.68	1023 ± 81	380	37.15
Feb. 7.....	3501 ± 263	1235	35.28	1100 ± 106	497	45.18
Feb. 14.....	3070 ± 225	1056	34.40	1201 ± 134	627	52.21
Feb. 21.....	4671 ± 496	2324	49.75	1218 ± 148	693	56.90
Feb. 28.....	2507 ± 151	708	28.24	1145 ± 101	474	41.40
Mar. 6.....	2934 ± 183	859	29.28	1117 ± 76	354	31.69
Mar. 13.....	2532 ± 221	1037	40.96	1183 ± 138	648	54.78
Mar. 20.....	2563 ± 166	779	30.39	1084 ± 83	389	35.89
Mar. 27.....	3044 ± 217	1015	33.34	1112 ± 104	488	43.88
Apr. 3.....	3322 ± 335	1571	47.29	1181 ± 127	595	50.38
Apr. 10.....	3966 ± 337	1578	39.79	1027 ± 123	576	56.09
Apr. 17.....	3734 ± 245	1149	30.77	1107 ± 121	569	51.40
Apr. 24.....	3073 ± 288	1352	44.00	933 ± 134	627	67.20
May 1.....	4165 ± 316	1483	35.61	933 ± 93	438	46.95
May 8.....	3358 ± 251	1178	35.08	1092 ± 87	407	37.27
May 15.....	3775 ± 190	891	23.60	1071 ± 100	468	43.70
May 22.....	3624 ± 169	792	21.85	1245 ± 110	516	41.45
May 29.....	4182 ± 324	1521	36.37	1410 ± 294	1377	97.66
June 5.....	4602 ± 205	959	20.84	1543 ± 169	790	51.20
June 12.....	4455 ± 473	2219	49.81	1270 ± 117	546	42.99
June 19.....	3376 ± 292	1371	40.61	1106 ± 114	536	48.46
Mean.....	3186 ± 83	731	22.94	1125 ± 16	139	12.36
Minimum.....	1861			864		
Maximum.....	4671			1543		

* October 24 and 26.

** November 4 and 9.

TABLE II

Group II—10 Rabbits. Consecutive Values for Lymphocytes and Monocytes

Date	Lymphocytes			Monocytes		
	Mean values	Standard deviation	Coefficient of variation	Mean values	Standard deviation	Coefficient of variation
<i>1928</i>	<i>per cmm.</i>	<i>per cmm.</i>	<i>per cent</i>	<i>per cmm.</i>	<i>per cmm.</i>	<i>per cent</i>
Mar. 29.....	2210 \pm 173	809	36.61	573 \pm 35	165	28.80
Apr. 3.....	3313 \pm 262	1227	37.04	677 \pm 101	474	70.01
Apr. 10.....	3073 \pm 287	1347	43.83	844 \pm 89	416	49.29
Apr. 17.....	3429 \pm 286	1342	39.13	1076 \pm 154	724	67.29
Apr. 24.....	3067 \pm 203	950	30.97	853 \pm 102	476	55.80
May 1.....	3257 \pm 231	1081	33.19	680 \pm 64	299	43.97
May 8.....	3215 \pm 216	1012	31.48	777 \pm 83	388	49.94
May 15.....	2605 \pm 247	1158	44.45	721 \pm 126	590	81.83
May 22.....	3960 \pm 284	1331	33.61	1028 \pm 101	475	46.21
May 29.....	3758 \pm 365	1713	45.58	843 \pm 97	455	53.97
June 5.....	3864 \pm 252	1180	30.54	796 \pm 70	329	41.33
June 12.....	3236 \pm 244	1144	35.35	1063 \pm 110	517	48.64
June 19.....	3526 \pm 222	1040	29.50	1166 \pm 66	310	26.59
Mean.....	3270 \pm 87	465	14.22	854 \pm 32	173	20.26
Minimum.....	2210			573		
Maximum.....	3960			1166		

TABLE III

Group III—10 Rabbits. Consecutive Values for Lymphocytes and Monocytes

Date	Lymphocytes			Monocytes		
	Mean values	Standard deviation	Coefficient of variation	Mean values	Standard deviation	Coefficient of variation
<i>1928</i>	<i>per cmm.</i>	<i>per cmm.</i>	<i>per cent</i>	<i>per cmm.</i>	<i>per cmm.</i>	<i>per cent</i>
Sept. 20.....	1971 \pm 268	1254	63.62	712 \pm 113	529	74.30
Sept. 28.....	2454 \pm 287	1345	54.81	780 \pm 88	414	53.08
Oct. 10.....	2178 \pm 82	385	17.68	739 \pm 80	373	50.47
Oct. 19.....	2497 \pm 142	667	26.71	813 \pm 103	482	59.29
Nov. 2.....	2009 \pm 167	784	39.02	797 \pm 101	472	59.22
Nov. 9.....	2352 \pm 151	708	30.10	820 \pm 64	300	36.59
Nov. 16.....	1883 \pm 110	516	27.40	733 \pm 89	415	53.69
Nov. 22.....	2348 \pm 181	850	36.20	895 \pm 112	525	58.66
Mean.....	2212 \pm 53	220	9.95	786 \pm 13	55	7.00
Minimum.....	1883			712		
Maximum.....	2497			895		

values, together with the probable errors of the means, the standard deviations, and the coefficients of variation. The curves in Text-figs.

TABLE IV

Group IV—10 Rabbits. Consecutive Values for Lymphocytes and Monocytes

Date	Lymphocytes			Monocytes		
	Mean values	Standard deviation	Coefficient of variation	Mean values	Standard deviation	Coefficient of variation
1928-29	per cmm.	per cmm.	per cent	per cmm.	per cmm.	per cent
Nov. 27.....	2616 \pm 173	812	31.02	546 \pm 39	183	33.56
Dec. 4.....	2713 \pm 153	717	26.44	499 \pm 45	212	42.47
Dec. 11.....	2319 \pm 182	854	36.84	452 \pm 55	256	56.63
Dec. 18.....	2168 \pm 211	991	45.72	790 \pm 171	801	101.35
Dec. 26.....	2160 \pm 186	871	40.31	362 \pm 49	229	63.24
Jan. 2.....	1563 \pm 82	383	24.48	456 \pm 60	280	61.40
Jan. 8.....	2040 \pm 172	807	39.55	490 \pm 90	422	86.08
Jan. 15.....	1955 \pm 84	394	20.13	563 \pm 48	224	39.80
Jan. 22.....	1839 \pm 160	751	40.81	704 \pm 87	406	57.72
Jan. 29.....	2470 \pm 154	720	29.15	591 \pm 73	342	57.84
Feb. 5.....	1873 \pm 144	676	36.09	546 \pm 70	330	60.34
Feb. 13.....	2036 \pm 108	505	24.80	826 \pm 148	693	83.89
Feb. 19.....	1907 \pm 146	686	35.97	893 \pm 101	474	53.08
Feb. 26.....	2038 \pm 139	651	31.96	1066 \pm 100	468	43.87
Mar. 12.....	2007 \pm 142	668	33.29	1006 \pm 92	431	42.81
Mar. 19.....	2189 \pm 129	604	27.58	857 \pm 74	346	40.40
Mar. 26.....	2290 \pm 199	934	40.76	635 \pm 66	310	48.85
Apr. 2.....	1561 \pm 185	869	55.70	856 \pm 94	443	51.74
Apr. 9.....	2530 \pm 168	787	31.09	1066 \pm 98	460	43.15
Apr. 16.....	2765 \pm 233	1092	39.50	709 \pm 57	269	37.95
Apr. 23.....	2971 \pm 200	939	31.62	916 \pm 99	462	50.44
Apr. 30.....	2301 \pm 183	857	37.23	619 \pm 88	413	66.63
May 7.....	2574 \pm 146	686	26.64	699 \pm 46	216	30.95
May 14.....	2514 \pm 150	668	26.55	622 \pm 62	276	44.34
May 21.....	1672 \pm 93	413	24.71	565 \pm 36	159	28.19
May 28.....	2523 \pm 169	752	29.78	479 \pm 58	259	54.11
June 4.....	1960 \pm 172	763	38.94	678 \pm 62	276	40.77
June 11.....	1920 \pm 153	682	35.53	509 \pm 44	196	38.50
June 18.....	2609 \pm 205	860	32.94	530 \pm 42	176	33.18
Mean.....	2210 \pm 45	363	16.40	673 \pm 24	190	28.20
Minimum.....	1561			362		
Maximum.....	2971			1066		

1 and 2, illustrating the mean values, have been drawn in the form of the percentage deviations of the smoothed means from standard

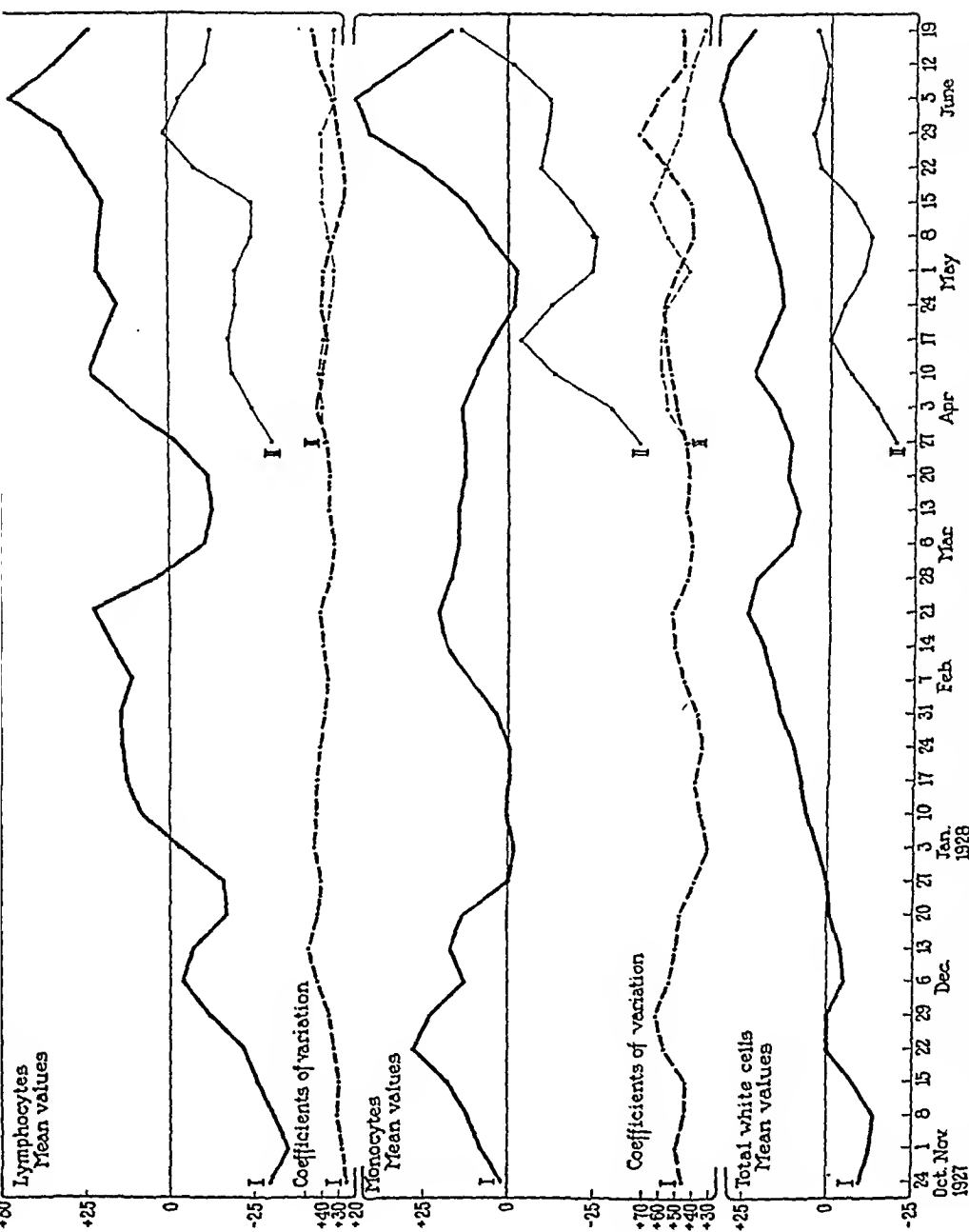
values (1,4); similar curves for the total white cells (2) have been included to facilitate convenient comparisons with their fluctuations. The other curves in the text-figures represent the smoothed coefficients

TABLE V

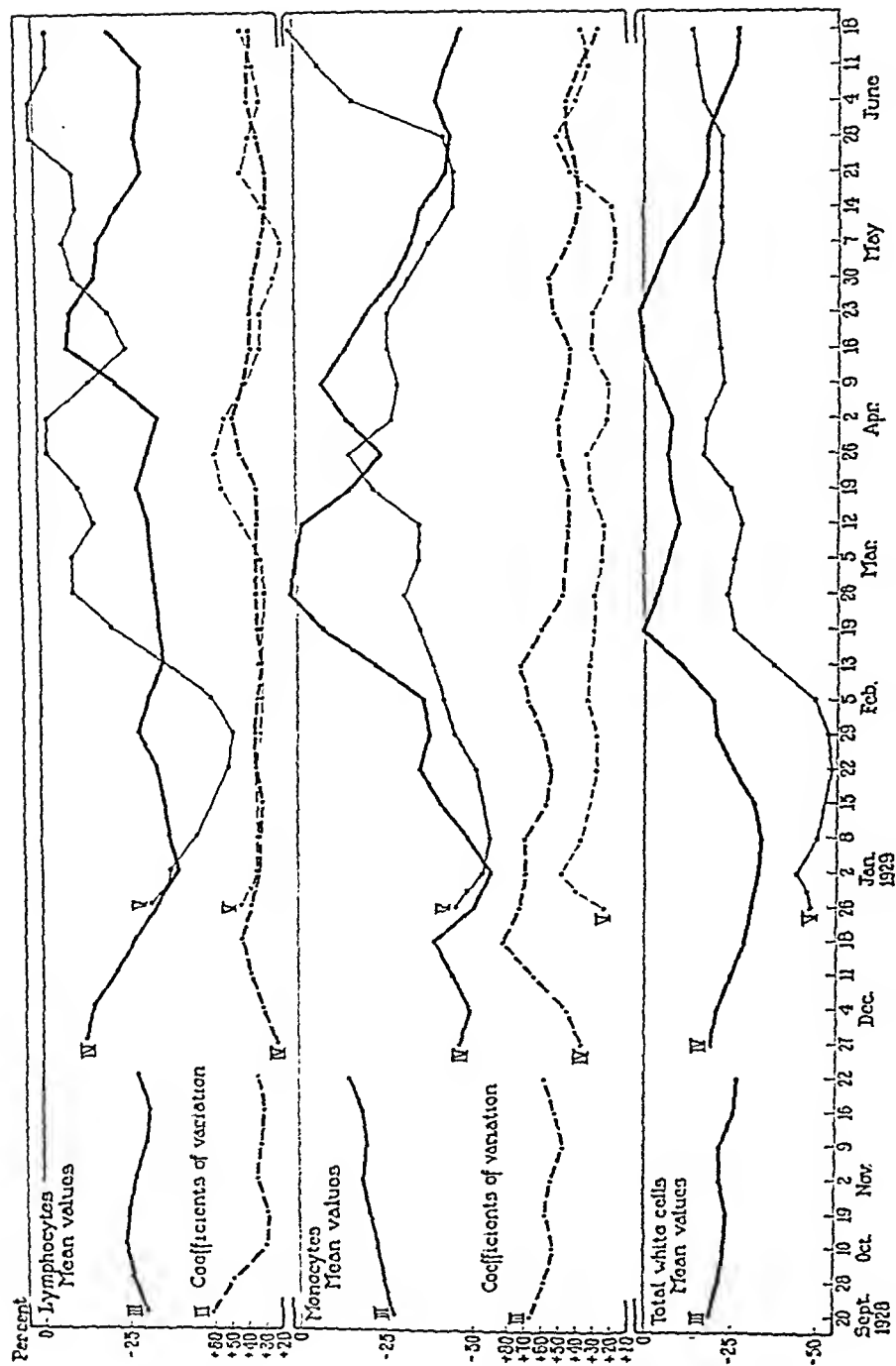
Group V—5 Rabbits. Consecutive Values for Lymphocytes and Monocytes

Date	Lymphocytes			Monocytes		
	Mean values	Standard deviation	Coefficient of variation	Mean values	Standard deviation	Coefficient of variation
1928-29	per cmm.	per cmm.	per cent	per cmm.	per cmm.	per cent
Dec. 29	2310 \pm 95	314	13.59	631 \pm 92	305	48.34
Dec. 31	1650 \pm 206	683	41.39	350 \pm 34	113	32.29
Jan. 3	2127 \pm 386	1279	60.13	588 \pm 65	215	36.56
Jan. 10	1676 \pm 130	431	25.72	309 \pm 26	86	27.83
Jan. 24	1135 \pm 107	353	31.10	544 \pm 60	200	36.76
Jan. 31	1503 \pm 90	299	19.89	505 \pm 46	153	30.30
Feb. 7	1198 \pm 139	460	38.40	591 \pm 44	146	24.70
Feb. 15	2147 \pm 211	698	32.51	605 \pm 69	227	37.52
Feb. 21	2304 \pm 146	485	21.05	618 \pm 47	156	25.24
Mar. 1	2941 \pm 328	1087	36.96	723 \pm 71	234	32.37
Mar. 8	2820 \pm 156	517	18.33	639 \pm 47	146	22.85
Mar. 15	2387 \pm 167	552	23.13	561 \pm 67	223	39.75
Mar. 22	2610 \pm 214	710	27.20	816 \pm 148	492	60.29
Mar. 29	3092 \pm 396	1314	42.50	905 \pm 133	441	48.75
Apr. 5	3038 \pm 133	440	14.48	671 \pm 132	436	64.98
Apr. 12	2668 \pm 114	377	14.13	635 \pm 54	179	28.19
Apr. 19	1990 \pm 226	749	37.64	875 \pm 73	242	27.66
Apr. 26	2357 \pm 208	688	29.19	523 \pm 60	199	38.05
May 3	2927 \pm 160	529	18.07	788 \pm 31	101	12.82
May 10	2700 \pm 117	388	14.37	563 \pm 31	101	17.94
May 17	2886 \pm 139	459	15.90	524 \pm 32	106	20.23
May 24	2219 \pm 184	610	27.49	547 \pm 85	283	51.74
May 31	3580 \pm 605	2006	56.03	531 \pm 56	184	34.65
June 7	2913 \pm 176	584	20.05	880 \pm 60	198	22.50
June 14	2913 \pm 173	572	19.64	1023 \pm 107	353	34.51
June 21	2944 \pm 260	861	29.25	1007 \pm 125	414	41.11
Mean	2424 \pm 80	607	25.04	652 \pm 23	176	26.99
Minimum	1135			350		
Maximum	3580			1023		

of variation of the means. The standard values used in this comparison of mean values are: lymphocytes, 3050 per cubic millimeter; monocytes, 1000 per cubic millimeter; total white blood cells, 9560 per cubic millimeter.



TEXT-FIG. 1. Mean values for consecutive lymphocyte and monocyte determinations as percentage deviations from standard values. 1927-28.



TEXT-FIG. 2. Mean values for consecutive lymphocyte and monocyte determinations as percentage deviations from standard values. 1928-29.

DISCUSSION AND SUMMARY

The results of these experiments will be discussed in chronological order. It will be noted that the observations on Group II were made during the spring of 1928 at which time Group I was still being examined, and that Groups IV and V were followed during the winter and spring months of 1928 and 1929.

Group I was examined at weekly intervals from October 24, 1927 to June 24, 1928 (Table I). From the curves in Text-fig. 1, which illustrate the lymphocyte and monocyte mean values in the form of percentage deviations from standard values as given above, it will be seen that the mean numbers of lymphocytes were considerably increased above their low initial level by the end of the experiment. This change was accomplished gradually and in a fairly consistent fashion with the exception of a minor drop of the values in December and June and an abrupt and pronounced fall at the end of February and the first half of March. In the case of the monocytes, the general level of which was continuously above the standard value, no such sustained increase of mean values was observed. The curve representing these cells contains 3 major upward swings which occurred in November, in February and March, and in May. During certain periods, as in October and November, and again in May and June, the lymphocyte and monocyte curves follow the same general trend. At other times, however, this parallel relationship did not hold. In January, the lymphocyte curve describes a sharp rise while the monocyte curve is stationary and in February, March, and April, when an abrupt fall of the lymphocyte curve is followed by a pronounced rise, the monocyte curve describes a slight but protracted decline. It will be seen by the curve in Text-fig. 1, that the total white cell count was considerably increased during the experiment, and a comparison with the lymphocyte curve shows that its fluctuations are frequently reflected in those of the total white cells. Although the coefficients of variation of the lymphocyte means (Table I) are of a high order of magnitude, the curve of the smoothed values (Text-fig. 1) is quite regular. The monocyte coefficients (Table I) are slightly higher than those of the lymphocytes and their smoothed curve (Text-fig. 1) is more irregular.

Group II was examined from March 29 to June 19, 1928 (Table II). The curves representing its lymphocyte and monocyte means (Text-fig. 1) bring out clearly the lower numerical levels of both classes of cells as compared with those of Group I. In the case of the lymphocytes the general forms of the curves of Groups I and II are very similar but with the monocytes, the resemblance is less striking.

The lymphocyte and total white cell curves are, on the whole, much alike. There are certain portions of the latter curve, however, which resemble the monocyte more than the lymphocyte curve, as for example, that covered by the last two observations. The coefficients of variations of the lymphocyte and monocyte means of Group II (Table II) are of the same order of magnitude as those of Group I and their smoothed curves (Text-fig. 1) present the same general appearance.

Group III was examined from September 20 to November 22, 1928 (Table III). From the curves in Text-fig. 2, it will be seen that the levels of both the lymphocyte and the monocyte mean values were of the same order as those of Group II rather than those of Group I. Only minor changes in the values of Group III were observed. The monocyte curve describes a gradual comparatively slight rise while the lymphocyte curve shows no clearly defined trend. The total white cell curve describes a slight downward trend and it is almost a mirror image of the monocyte curve. With the exception of the first two points of the lymphocyte curve, the curves of the smoothed coefficients of variation of the lymphocyte and monocyte means (Text-fig. 2) are comparable to those of Groups I and II.

Group IV was examined from November 27, 1928 to June 19, 1929 (Table IV). The general level of the lymphocyte mean values was comparable to the results in Groups II and III and to the first quarter of the findings of Group I. During December, the trend of mean values was decreasing as shown by the falling curve (Text-fig. 2); during January, February, and March, a low level was fairly constantly maintained; in April, an abrupt increase occurred and this was succeeded by a second decrease. The curve representing the monocytes (Text-fig. 2) shows first that the general level of mean values was usually lower than that of Group I and was more comparable to those of Groups II and III. From very low levels in December and early January, the pronounced upward swing of the curve in February, March, and April indicates the increase of monocytes during this time, and its subsequent descent illustrates the eventual drop in the numbers of these cells. The general trend of the lymphocyte and the monocyte means is similar in the beginning and during the last third

of the experiment, but during much of the time, the increase of monocytes was accompanied by practically no change in the level of lymphocyte mean values. In general form, the total white cell curve (Text-fig. 2) has many points of resemblance with the monocyte as well as with the lymphocyte curve.

The coefficients of variation of the lymphocyte means (Table IV, Text-fig. 2) which are on the whole quite uniform, are of a similar order of magnitude to those of the other groups. The coefficients of the monocytes (Table IV, Text-fig. 2) are somewhat more variable and tend to be slightly higher than those of the other groups.

Group V was examined from December 29, 1928 to June 21, 1929 (Table V). The mean lymphocyte values as shown by the curve in Text-fig. 2 were on the whole higher than those of Group IV examined during the same months, but they did not equal the high levels of Group I examined the previous year. In its first portion, the curve representing these findings resembles that of Group IV, but its rise was initiated in February and from then onward to the end of the experiment, a comparatively high level prevailed. The rise of the Group IV curve, which was of short duration, occurred in March and April. In respect to the maintenance of higher values in the spring months, Group V resembled Groups I and II. The monocyte curves of Groups IV and V are, on the whole, similar in general form until the end of the experiment (Text-fig. 2); during June, the curve for Group V describes an abrupt and marked rise while that of Group IV continues its downward trend. The general level of the Group V mean values was slightly lower than those of Group IV. It will be noted by comparing their respective curves, that the movements of the lymphocyte and monocyte mean values are generally similar in direction during the first two-thirds of the experiment. In April and May, however, the lymphocyte curve is rising while the monocyte curve is falling; in June the lymphocyte curve is stationary at a high level while the monocyte curve shows an abrupt rise. On the whole, the general contour of the total white cell curve of Group V (Text-fig. 2) resembles the lymphocyte more than the monocyte curve. During April and May, however, when its level continues to be quite regularly maintained, the lymphocytes are rising and the monocytes

are falling; in June, when it is undergoing a gradual and comparatively slight rise, the lymphocytes are high and the monocytes are very high.

The coefficients of variation of the lymphocyte means of Group V (Table V, Text-fig. 2) are entirely comparable to those of Group IV; those of the monocyte means (Table V, Text-fig. 2) on the other hand, are considerably smaller and are generally more uniform. As far as general level is concerned, the monocyte coefficients of Group V are the lowest of any of the 5 groups.

There are certain features of these results which merit special attention. In the first place, the general levels of both lymphocyte and monocyte mean values during 1927-28 were on the whole higher than in 1928-29, as was found with the red cells and hemoglobin (1), the total white cells (2), and the neutrophils, the basophiles, and the eosinophiles (3). Secondly, the parallelism shown by two groups examined during the same months with respect to the direction of a change in the level of mean cell values and the time of its occurrence, which was characteristic of the other cells and of the hemoglobin content, was less pronounced in the case of the lymphocytes and monocytes. With Groups I and II, the lymphocyte curves are very similar but those of the monocytes are less alike; with Groups IV and V, the monocyte curves up to their last periods have a general resemblance to each other while the lymphocyte curves are frequently dissimilar. Thirdly, the general trend of cell level shown by the lymphocytes was one of increasing values in the late winter and spring months while lower values prevailed in the fall and winter. In the case of the monocytes, a similar but less consistent trend toward higher numerical values was observed and in addition to a late winter and spring rise, there was some indication that higher counts were characteristic of the early winter months (Group I, November; Group III, rise of curve from a lower September to a higher November level; Group IV, fall of curve from a higher November to a lower December level). Fourthly, the period of greatest irregularity in the lymphocyte mean values occurred in the late winter and spring months of both years; with the monocytes, on the other hand, this feature was observed in the fall and early summer months of the first year and during the late winter, the spring and the early summer months of the second year.

The significance to be attached to the major changes in the levels of lymphocyte and monocyte mean values is indicated by the following examples of the ratios of the differences of various means to their probable errors.

Group number	Lymphocytes			Monocytes		
	Dates of mean values		Ratio	Dates of mean values		Ratio
I	Oct. 24	June 19	3.26	Oct. 24	June 5	2.94
	Feb. 21	May 20	4.03	May 1	June 5	3.16
	Mar. 20	June 5	7.72			
II	Mar. 29	May 22	5.26	Mar. 29	June 19	7.91
III	Sept. 20	Oct. 19	1.74	Sept. 20	Nov. 22	0.92
IV	Nov. 27	Jan. 2	6.61	Dec. 26	Feb. 26	6.34
	Jan. 2	Apr. 23	3.45	Feb. 26	June 18	4.96
	Apr. 23	June 11	2.46	Feb. 26	Mar. 26	3.59
V	Dec. 29	June 14	3.06	Dec. 26	June 21	2.43
	Jan. 24	Mar. 8	8.92	May 17	June 14	4.46
	Apr. 19	June 7	3.23			

The question of the numerical relationship of lymphocytes and monocytes in the peripheral blood has received much attention. In so far as the results on these groups of normal rabbits are concerned, the present findings indicate that a constant and consistent relationship between these cells does not obtain over long periods of time. This feature of the observations may be best appreciated by comparing the respective curves representing the consecutive weekly mean values of these cells (Text-figs. 1 and 2). In certain portions, both curves show similar changes in level during the same weeks, indicating that both classes of cells have undergone analogous numerical changes (Group I, November, May, June; Group IV, January, April, May; Group V, February, April). In other portions, however, the curves move in opposite directions (Group I, December, January, April; Group II, June; Group III, October; Group IV, December, February; Group V, May, June). It is evident, however, that before final conclusions on the lymphocyte-monocyte numerical relationship can

be drawn, the results of experiments of this type must be analyzed from the standpoint of the individual animal.

CONCLUSIONS

Consecutive weekly observations on the lymphocyte and monocyte counts of the peripheral blood were made on 5 groups of normal rabbits, a total of 45 animals, during a period of 20 months from October, 1927 to July, 1929. Individual groups were examined 8 to 35 weeks. The results are analyzed on the basis of the mean group values of each week.

In the case of the 4 groups followed 13 to 35 weeks, there was a general tendency for the lymphocyte mean values to become increased; with the group observed 8 weeks, the level of mean values showed little change. The general trend of the monocyte mean values was also in the direction of higher levels but it was less pronounced than that of the lymphocytes.

The period of greatest irregularity in the mean values of the lymphocytes was in the late winter and spring months of both years. With the monocytes, periods of fluctuating values occurred in the fall of 1927, the spring and early summer of 1928, and in the late winter, spring, and early summer of 1929.

There was a certain degree of parallelism in the case of two groups examined during the same months with respect to the direction and time of occurrence of a change in the level of lymphocyte and monocyte mean values.

The general levels of lymphocyte and monocyte mean values in the groups examined during 1927-28 were higher than in the groups of 1928-29.

The results based upon the trends of mean group values obtained from consecutive weekly observations showed no evidence of a consistent numerical relationship between lymphocytes and monocytes.

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EXPERIMENTAL NEPHRITIS IN THE FROG

I. THE ANATOMICAL EVIDENCE OF DAMAGE*

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PLATES 7 TO 9

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In the complex problems which have arisen in the study of nephritis the experimental method has proved a useful adjunct. Many aspects of the disease which can be examined only at the end of long continued processes in man and which, therefore, remain obscure to purely anatomical investigation, become relatively simple when examined during their development by this method which allows freedom of procedure and more rigid control to the investigator.

In one aspect, however, the advance has not been encouraging, and that is the correlation of disturbances in the function of the organ with changes in its anatomical structure. Various reasons are evident for this situation. The normal function of the kidney is far from understood, and this is particularly true of the complex kidney of mammals. A further complicating factor is that the nephritis which develops under experimental conditions is of almost as involved a nature as that found in the disease of man.¹ The first and great advantage of the experi-

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¹ An example of this is found in the different interpretations which have been offered for the histiogenesis of experimental chronic uranium nephritis in mammals. Dickson (*Arch. Int. Med.*, 1909, 3, 375; 1912, 9, 557) believes the chronicity of the lesion to be the result of a vascular lesion; Suzuki and Aschoff (*Verhandl. Deut. path. Gesell.*, 1912, 15, 199) to a hydronephrotic factor, and Oliver (*Journ. Exp. Med.*, 1915, 21, 425; 1916, 23, 301) to a direct inflammatory stimulation of the connective tissue.

mental method, namely, the simplification and control of conditions, is not therefore really available in its full extent when mammals are subjected to its procedures.

An analogous situation has been recognized by the physiologists in their work and these difficulties met by modification of the direction of their attack. The work of Richards (1), Bieter and Hirschfelder (2), Marshall (3), and Höber (4), to mention but a few, in which direct methods have been applied to simple kidneys has objectively established a few certain facts and thus removed them from the field of hypothesis where they had so long remained. The application of these newer methods to the study of the abnormal kidney is an obvious step. Direct experimentation, as Richards has termed it, both by visual observation of the living functioning organ and by perfusion of it, a method so brilliantly used by Höber and his pupils, might be used with the simple kidney of the frog in which abnormalities have been produced. It is possible that this might allow of the establishment of a base from which a later extension to the more complicated conditions obtaining in the abnormal mammalian kidney could be taken.

The present investigation is the first of a series of such studies of experimental nephritis in the frog.² As a first step, the method of production of the experimental lesions in the frog's kidney is described and the types of nephritis which may be produced in the animal detailed. In subsequent articles changes in the circulatory reactions of kidneys in which an experimental nephritis has been produced will be described and contrasted with those of the normal kidney. The response of the kidney, as tested by the method of perfusion, to damage of limited extent and of controlled nature as it may be produced in the isolated organ will next be given. The final step will be the presentation of a similar functional study of the disease as it exists when produced in the living frog and a correlation of these changes with the anatomical findings in the light of what we have learned with this "artificial" nephritis in the isolated organ.

² Four studies preliminary to this series, concerned with certain physiological aspects of our problem have appeared in the *Journal of Experimental Medicine*, 1929, 50, 15; 1929, 50, 601; 1930, 51, 161; and the *American Journal of Physiology*, 1930, 93, 363.

Methods

The animals used were chiefly *R. catesbiana*, since their kidneys are large and show the lesions most satisfactorily.

In our choice of renal poisons we have been guided by past studies of the similar problem in mammals. The list of agents we have used are: corrosive sublimate, potassium bichromate, uranium nitrate, ricin, cantharidin and snake venom. The first three substances have been classified from the lesions which they produce in mammals as tubular poisons, while the last three are reported to affect with more or less specificity the vascular apparatus of the kidney.

The experimental procedure was as follows.

Various amounts of a substance were injected into the dorsal lymph sacs of the frogs and the animals returned to their water tanks. After definite periods of time they were killed and portions of their kidneys fixed in 10 per cent formalin, Orth's fluid, Bensely's mixture, and Kolster's fixative. Sections were stained with hematoxylin and eosin, Van Gieson's mixture, iron hematoxylin, Mallory's connective tissue stain, Weigert's fibrin stain and for the mitochondria by Bensely's and Kolster's methods. In all cases great care was taken to fix the kidney tissue promptly after death. Post mortem change is extremely rapid if the frog lies dead in the tank and the autolytic changes resemble somewhat those which may occur as a result of toxic substances. For this reason frogs found in the morning, which might possibly have died earlier in the night were discarded and dying animals were killed as soon as their condition was detected. An apparent variation was frequently noted in the toxicity of a given substance when administered to different frogs. This we think was the result of diffusion of the substance from the dorsal lymph sac of the frog through the puncture wound into the water of the tank. Such irregularities may be largely avoided by intraperitoneal injection but the possibility of their occurrence must be kept in mind in the comparison of lesions in different animals.

We would call attention to the fact that the frog may suffer from spontaneous lesions of the kidney. These are the result of a general parasitic infection of the animal in which focal lesions develop in the kidney as well as in other organs. These lesions, consisting of circumscribed round areas of necrosis filled with nuclear debris which involve all the tissue cells indiscriminately in the infected focus, may be easily recognized. All frogs which were found to be so infected were discarded.

EXPERIMENTAL

The Lesion with "Vascular" Poisons

We were unable to produce constant lesions in the kidneys of the frogs which had been injected with the so-called vascular poisons. In the case of cantharidin the insolubility of the substance in water requires a solvent, acetic ether, which is even more toxic than the drug. The animals succumbed in a short time to the injection 0.5 cc. and if given in lesser amounts so little cantharidin was administered that no ill effects were noted after repeated injections. Large repeated injections of ricin, totaling 10 cc. of a saturated saline solution in the course of 3 days, produced no ill effects and the kidneys appeared entirely normal. The animals were also resistant to the action of snake venom. Doses which would be enormous for mammals did not kill the frogs, and when death followed repeated injections the kidneys showed no definite or constant effects.

These experiments, on account of their negative findings, are not described in detail but may be summarized by the statement that we were unable to produce lesions with regularity by the use of these substances.

*The Lesion with "Tubular" Poisons**Corrosive Sublimate*

The amount of corrosive sublimate injected into the animal varied from 1 to 7.5 mg. The average weight of the frogs being 500 gm. this equals an average dosage of 8 mg. per kilo. As mentioned before considerable variation in the apparent effect of the drug was noted but these irregularities were avoided by disregarding those animals in which the experiment had failed to damage the kidneys.

As in mammals a striking feature of the action of the sublimate was the damage to the tubular epithelium (Fig. 1).

This was apparent within 18 hours following the injection and in this early period consisted of marked cloudy swelling of the tubular epithelium with occasional necrosis and desquamation. The seat of the lesion was definitely limited to

the broad proximal convoluted segment of the renal tubule, the narrow neck, distal convoluted portion and the collecting tubules remaining essentially normal.³

In the mitochondrial preparations the usual evidences of early cellular damage were found. The granules in the involved portion of the tubule were swollen and irregularly distributed in agglutinated clumps and masses. In some necrotic cells these irregular clumps of heavily stained material persisted while in others solution of the substance had occurred so that the dead cell body was free of any granular substance. In these early stages of the damage the batonnets of the distal convoluted tubule stained normally.

In striking contrast to the effects of sublimate on mammals was the early and constant evidences of damage which were observed in the glomeruli.

In many cases these lesions were present in kidneys whose tubules showed little if any abnormalities and they were constantly found in those where the damage was at all severe. The simplest abnormality observed was the deposit of granular material in Bowman's space. This material was in some cases arranged in fibrin-like threads but its staining reaction was not that of typical fibrin. In other cases this material was condensed and deposited on the surface of the glomerular tuft where it formed a thin dense covering which followed the indentations between the capillaries into the central portions of the structure. Such appearances were, however, usually noted in kidneys which were more severely damaged than those which we are at present describing. Beside these lesions in the tubules and glomeruli the interstitial tissue showed a slight edema, and some general hyperemia of the capillaries between the tubules was occasionally present.

In animals which had received the larger doses and which had lived 70 hours the lesions were much further developed and consequently were more severe.

Extensive necrosis of the tubular epithelium was present and the desquamated dead cells filled the lumen of the proximal convoluted tubule. This portion in fact no longer existed except as a space within the membrana propria which was packed solid with granular debris. The damage in such kidneys had also spread both back toward the glomerulus and in the opposite direction, so that examples might be found where the neck and the distal convoluted tubule were also destroyed. The collecting tubules were only slightly altered. In them, however, was found debris, evidently derived from higher reaches of the tubule, and here

³ We have used the terminology of Stewart (5) because of its analogies with that used for the mammalian kidney. Proximal convoluted tubule is synonymous with Segment II, distal tubule with Segment III, and collecting tubule with Segment IV of other writers.

also definitely formed casts were present. They were of the hyaline type as a rule, though some contained granular material. It was a striking finding that such fully formed consolidated casts were found almost only in the distal convoluted tubule and in the excretory ducts and were seldom seen in the proximal portion of the tubule where the active damage was occurring. For this reason in a frontal section through the kidney a peculiar distribution of them was found since they were clustered in those sections of the tubule that lay around the glomeruli in the middle and ventral portions of the kidney.

The glomeruli of such kidneys showed even greater damage than has been described. The same collections of fibrinoid substance in Bowman's space were found but in the tissues of the tuft definite changes were also observed. Intense engorgement with the formation of hyaline thrombi composed of agglutinated red blood cells and scattered areas of edema and even necrosis were present. Patches of nuclear débris were scattered among the loops and occasionally a tuft could be found so severely damaged that hemorrhage had occurred into the capsular space. On the other hand some damaged glomeruli were anemic, and might be found greatly altered and even disrupted without any escape of blood from their capillaries. These glomerular lesions were at times the most striking abnormality found in the kidneys. In the kidney of one animal which had received 1 mg. and was killed on the second day the tubules showed no very definite lesions, yet the glomeruli were severely involved.

In the tubules of the kidneys of animals which showed these severe glomerular lesions beside the hyaline and granular cast mentioned above, red blood cells were occasionally found either still discrete and in varying degrees of disintegration or consolidated into definite blood casts (Fig. 2).

Beginning about 3 or 4 days after the injection of the toxic substance reparative processes could be found in the kidneys.

These consisted of regenerative proliferation of the epithelium of the tubules which had been damaged. In the persisting cells, scattered through the region of the tubule which was most severely involved, and at the junction of this part with portions of the tubule which were relatively undamaged, mitotic figures were frequent. One such source of new formed cells was regularly found in the collecting tubules which were rarely damaged by the toxic agent. Cross sections of these tubules showing marked proliferative changes in the epithelium might be found scattered among the necrotic tubules of kidneys which had been so severely damaged as to show destruction of not only the proximal convoluted tubule but of the distal as well.

The new formed cells could be recognized by their unusual appearance. Their nuclei were large, often twice the size of those of the original tubule cell and were as a rule oval in shape. Their chromatin was increased in amount and stained quite heavily. Such nuclei were both excessive in number and irregularly dis-

tributed so that they formed irregular giant cell-like masses covered with protoplasm which projected irregularly into the lumen of the tubule. From such centers of proliferation the cells spread out over the denuded membrana propria. These new formed cells were found apparently creeping beneath detritis and casts which filled the tubule lumen so that in specimens 14 days old the lining of the tubules was found to be completely restituted, though the new cells could be still distinguished from the regularly arranged lining of the original epithelium.

Repair in the glomeruli seems to be a much slower process. The remains of the early lesions could still be made out after 14 days, a time when the tubular damage had been largely repaired. The more acute process of hyperemia had disappeared from the tuft but granular material and fibrinoid substance was still present in Bowman's space. Very occasionally mitotic figures were found in the tissues of the tuft, but no definite "chronic" lesions such as organization or definite proliferation of the tuft tissues were present.

Potassium Bichromate

The lesions after potassium bichromate resembled in a general way those following the administration of corrosive sublimate. Certain minor differences were observed, however, particularly in the early phases of the damage or in kidneys that had only been slightly affected. The amount of drug given varied from 0.5 to 5 mg. per animal so that the dosage varied around an average of 5 mg. per kilo.

In the kidneys of frogs which were killed within 24 hours after smaller doses or in animals which succumbed in 2 or 3 days but whose kidneys were but moderately damaged, only the earlier and milder types of degeneration were found in the tubular epithelium. The lumen of the tubule throughout its length was filled with granular detritis, the cells showed a cloudy swelling with the presence of an occasional pycnotic nucleus, but little evidence of frank necrosis was present. This cellular damage, as in the case of corrosive sublimate, was limited to the proximal convoluted tubule.

In kidneys showing such slight lesions, the glomeruli, however, were almost constantly involved. Granular or fibrinoid material in Bowman's space with desquamation of the capsular epithelium, hyaline thrombosis and occasional small areas of necrosis with hemorrhage into the capsular space could be found (Fig. 3). In the interstitial tissue hyperemia was often intense, the capillaries between the tubules being crowded with red blood cells. Only a moderate number of casts of the hyaline type were present in the distal convoluted tubule and in the collecting tubules, though granular material was present throughout the entire renal unit. Occasionally collections of red blood cells still discrete or partially disintegrated could be found in the tubule lumen or were definitely formed into blood casts.

In more severely damaged kidneys the microscopic picture resembled even

more closely that seen after corrosive sublimate, for tubular damage was now equally severe, the entire proximal convoluted portion being necrotic and the tubule transformed into a mass of dead desquamated cells. This damage also spread both into the narrow neck and into the distal convolution. The glomerular lesions, exudation of granular and fibrinoid material, necrosis and hemorrhage, were more pronounced and casts of all sorts were present in the lumina of the tubules (Fig. 4).

Beginning at the third or fourth day regenerative changes in the damaged tubules were apparent. Their course was similar to that seen after sublimate, large irregular masses of epithelium filling the tubule lumen with giant cell-like structures. Mitotic figures were frequent among their large oval nuclei (Fig. 5). Repair was only slight in the glomeruli and in most cases proliferative changes could not be definitely determined.

The lesions after bichromate poisoning therefore differ somewhat from those following the administration of sublimate in that although the glomerular damage was equally intense the tubular lesions were somewhat slower to develop. In the end, however, they became equally severe.

Uranium Nitrate

After the administration of from 5 to 10 mg. of the uranium nitrate, averaging 15 mg. per kilo, lesions developed resembling those following potassium bichromate more closely than those produced by corrosive sublimate. On account of this similarity they will be only briefly described.

In cases of mild damage or during the first 24 hours of the development of the lesion, the tubules showed only slight evidences of cellular damage such as cloudy swelling and occasional necrosis. The glomeruli were regularly involved by the milder changes which produced an accumulation of protein material in the capsular space (Fig. 6). As the poisoning became more severe or the slighter changes more fully developed, the tubular damage was more pronounced until it equalled that seen with potassium bichromate (Fig. 7). It was only occasionally, however, that the typical picture following corrosive sublimate could be obtained, that is, a complete destruction of the entire proximal convoluted tubule and the replacement of it by masses of dead disintegrating cells. The interstitial lesions of edema and hyperemia, though present, were also less pronounced.

In the severely damaged kidney, the appearance is approximately the common picture seen after all these toxic agents. The glomeruli were engorged, hyaline thrombi were present, small areas of necrosis of the tuft could be seen and hemorrhage into Bowman's space (Fig. 8). In the proximal convoluted portion definite

necrosis with desquamation filled the lumen of the tubule with débris, and casts, both granular and hyaline, were present in the distal convoluted and collecting tubules.

In animals which lived from 4 days to a week the irregular regenerative proliferation of the surviving epithelium in the damaged tubule was very apparent. In one animal which was killed 18 days after the administration of the poison the necrotic material and other evidences of early damage were almost entirely removed, only an occasional cast still persisting in the tubules, yet the abnormal condition of the proximal convoluted tubule was apparent in the excessive number of atypically new formed cells which formed its lining.

DISCUSSION

In a general way the lesions produced in the frog's kidney by potassium bichromate, corrosive sublimate and uranium nitrate are similar and resemble those which have been described as occurring in mammals. The significance of the differences observed, however, requires a brief discussion.

Of the tubular apparatus, in the frog as in the mammal, it is the proximal convoluted tubule which suffers most severely. For the latter group this point was first clearly established by Susuki (6) in his studies by means of vital staining. Although such special methods are required for the recognition of this point in the complex mammalian kidney, in the more simply arranged mesonephros of the frog the localization of the damage in the broad proximal convoluted tubule can be easily determined by means of its characteristic morphology and by the situation of this segment in the organ. As in the mammalian kidney, so too in the frog's mesonephros this limitation of the damage to a restricted portion of the tubule is observed only with small or moderate doses of the toxic agent. After the administration of large doses the damage spreads both toward the glomerulus and into the distal convoluted tubule. The straight portion of tubule connecting the two convoluted portions is thus involved as well in the lesion and though it can hardly be considered a loop of Henle, nevertheless its involvement in the spreading lesions is analogous to the fate of that structure under similar conditions in the mammal.

It is corrosive sublimate in the frog as in the mammal which produces the most extreme picture of tubular damage. We might point out, however, that the striking appearances seen after this substance

do not necessarily indicate a lesion of any greater severity than that which follows potassium bichromate or uranium nitrate where the histological picture is less impressive. In both cases the cells are killed but after sublimate the coagulation of the débris into massive blocks of deeply staining material contrasts with the disintegration of the dead cells which occurs after the administration of the other two poisons. The possibility of error in the estimation of the quantitative relation of the damage in the different forms of poisoning is therefore obvious.

In the effect of these "tubular" poisons on the glomeruli of the frog's kidney differences which at first hand may seem to be significant are observed. After all the poisons the glomeruli were regularly and definitely damaged. This damage was of equal degree to that seen in the tubules; in fact observations were recorded where the glomerular lesion was plainly evident in kidneys whose tubules showed very slight if any morphological evidence of involvement. The exudation of protein into Bowman's space in both granular and fibrinoid form, necrosis and thrombosis in the tuft tissues and hemorrhage from ruptured capillaries were regularly present. Such a regularity of striking morphological lesions has not been found in the mammalian kidney after similar treatment. It is a fact of great importance, nevertheless, that such lesions do occur though as a rule they are inconstant in occurrence or so slightly developed as to require some attention for their observation. After corrosive sublimate, both in animals and in fatal poisoning in man, it is not unusual to find protein material exuded into Bowman's space, and there is other evidence that the vessels throughout the kidney have also been damaged. Oertel (7) has emphasized the importance of these changes. Glomerular lesions of slight degree such as pycnosis of the nuclei of the tuft cells and the occurrence in them of hyaline degeneration have been described by Christian and O'Hare (8) as occurring occasionally after uranium poisoning. Furthermore the experiments of Wiesel and Hess (9) have shown that those glomeruli which may appear normal to morphological examination after the administration of uranium nitrate are in fact severely damaged, for a subsequent injection of adrenalin causes their weakened capillaries to burst and produces glomerular hemorrhages throughout the kidney.

It would seem, therefore, that the glomerular lesions which in mammals present only slight or inconstant morphological evidences of damage, are regularly observed, well developed and visible to simple methods of demonstration in the frog's kidney. The differences between the lesions in the two classes of animals is only a quantitative one, for qualitatively they are essentially similar.

The frog's mesonephros is particularly well suited therefore for the study of lesions which remain masked in the complex kidney of mammals. The importance of this point which forms the basis of our entire program of investigation will become increasingly apparent as the results of our investigations of nephritis in the frog by other methods are given. For this reason it is obviously too early a point in our work to discuss either the "nature" of the changes which are observed in these forms of kidney damage, or to attempt a classification of the toxic substances as either "tubular" or "vascular" since but one aspect of the problem, the morphological, has been given. The equally important functional manifestations of the lesions will be next considered.

SUMMARY

1. The damage produced by corrosive sublimate, potassium bichromate and uranium nitrate in the frog's kidney is described.

2. The morphological lesions consist of evidences of tubular damage, such as regressive changes in the epithelium, and of damage to the glomeruli ranging from increase in their permeability to gross damage of and hemorrhage from the tuft.

3. The point is emphasized that these lesions differ in their degree rather than in their nature from those found in the mammalian kidney after the administration of the same poisons.

4. The frog's kidney is exceptionally well suited therefore for the study of lesions which though present are masked in the complexities of structure and function of the mammalian kidney.

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EXPLANATION OF PLATES

Unless otherwise stated the sections of kidney were stained with Delafield's hematoxylin and eosine.

PLATE 7

FIG. 1. Kidney from an animal killed 48 hours after the injection of 5 mg. of corrosive sublimate. The section shows the central and dorsal zones (Stewart) of the kidney, the glomeruli and distal convoluted tubules lying in the former (upper half of section) and the broad proximal convoluted portions in the latter (lower portion of section). There is some granular material in the capsular spaces of the glomeruli. The cells of the proximal segment of the tubule are mostly necrotic and desquamated. Clustered around the glomeruli, the distal convoluted portions still show nuclear staining as do many of the straight portions which connect the two convoluted segments. 130 \times .

FIG. 7. Section shows the junction of the central and dorsal zones of the kidney of a frog which was killed 72 hours after the injection of 5 mg. of uranium nitrate. The capsular spaces of the glomeruli are filled with granular débris. There is widespread necrosis and desquamation of the epithelium of the proximal convoluted tubule in the lower part of the section, and somewhat less pronounced but similar lesions in the distal portion above. 130 \times .

PLATE 8

FIG. 2. The central zone of a kidney from an animal killed 72 hours after the injection of 1 mg. of corrosive sublimate. The section shows the glomeruli and surrounding distal convoluted segments. Although the epithelium of these portions is fairly well preserved many casts composed of disintegrated and agglomerated red blood cells are seen in their lumina. 120 \times .

FIG. 3. The junction of the central and ventral zones of the kidney of a frog killed 48 hours after the injection of 5 mg. of potassium bichromate. Lighter areas of necrosis containing nuclear débris are seen in the greatly swollen glomeruli. In spite of this glomerular damage the sections of tubules in the lower left corner, which are the terminal portions of the proximal convoluted tubule, show no definite lesions. 140 \times .

FIG. 4. Kidney of a frog killed 72 hours after the injection of 2 mg. of potassium bichromate. The section is stained with Mallory's connective tissue stain. Only a portion of the central zone of the kidney is shown. In this zone the distal convoluted tubules are filled with hyaline casts (deep blue). Covering the glomerular tuft and extending into it is a layer of fibrinoid material, which also stains deep blue rather than red as would true fibrin. 170 \times .

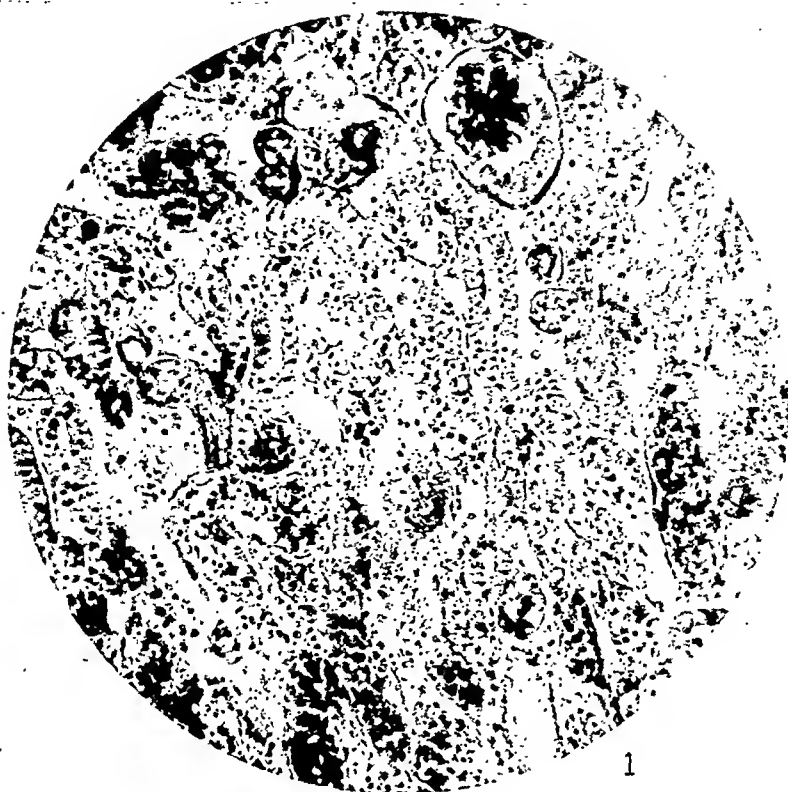
PLATE 9

FIG. 5. Section through the dorsal zone of a kidney of a frog which had been killed 120 hours after the injection of 5 mg. of potassium bichromate. Stained with iron hematoxylin after formalin fixation, a method which does not preserve the granular elements of epithelium. The lining of the proximal convoluted tubule, the only segment shown in the section, is composed of an excessive number of large regenerated cells whose nuclei are oval, and irregular in size, shape and distribution. A mitotic figure is seen in one of the upper cross sections. 500 \times .

FIG. 6. Section passing through the junction of the central and dorsal zones of a kidney from an animal which had been killed 72 hours after the administration of 5 mg. of uranium nitrate. The capsular space of all the glomeruli is filled with granular exudate. The cells of the distal convoluted tubules show moderate damage, but much less than the proximal convoluted segments which are not shown in the figure. 140 \times .

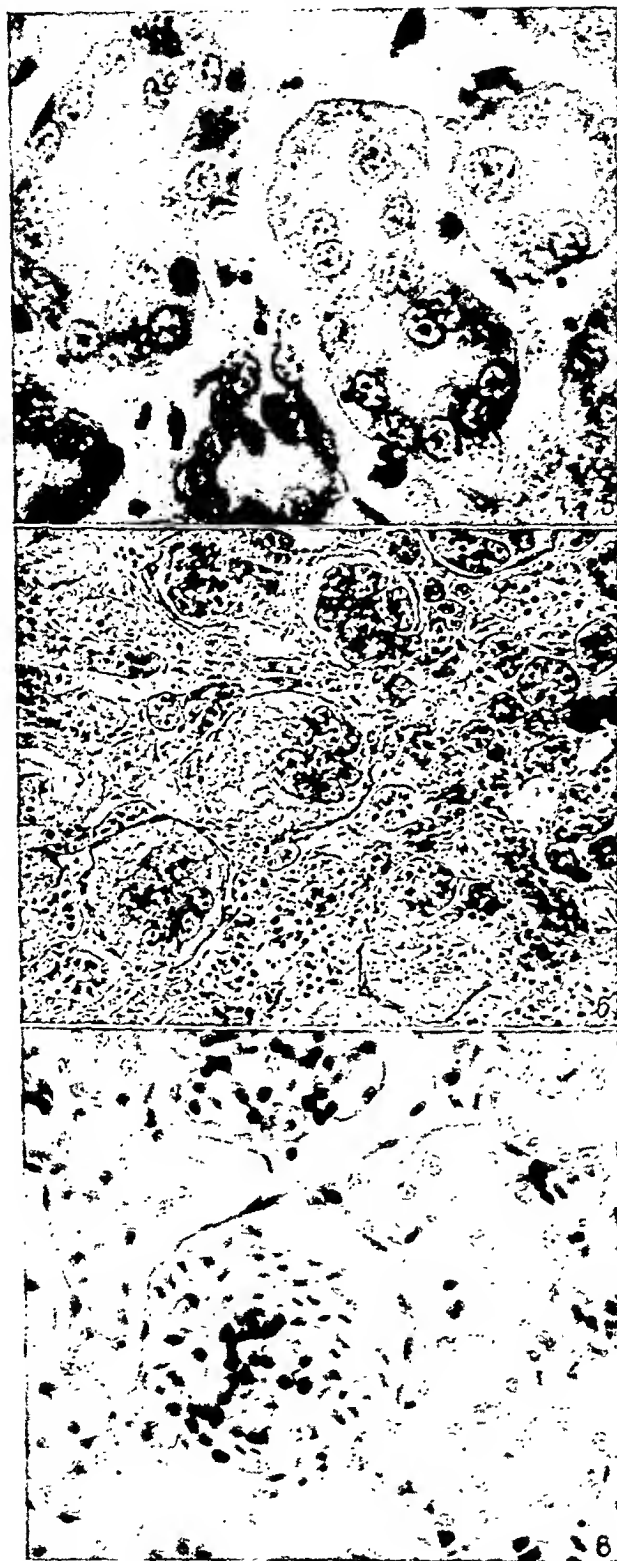
FIG. 8. Section shows a small portion of the central zone of a kidney from a frog killed 24 hours after the injection of 8 mg. of uranium nitrate. The sections through the distal convoluted tubules show them to be apparently normal. In the glomerular space is a recent hemorrhage from the tuft. The nucleated red blood cells are still well preserved. 300 \times .







(Oliver and Smith: Experimental neuritis in the frog. 1)



A STUDY OF THE RELATIONSHIP OF THE SCROTAL SWELLING AND RICKETTSIA BODIES TO MEXICAN TYPHUS FEVER

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In the study of typhus fever at the present time, etiological investigation turns to a considerable extent upon whether or not the small rickettsia-like bodies described in the scrotal swellings by Mooser can be looked upon as the causative agents, or whether they represent merely a secondary infection carried along through generations of guinea pigs together with the typhus virus.

Some light may be thrown upon this question by the occasional strains of Mexican typhus fever in which scrotal swelling does not occur for several guinea pig generations and then, for unknown reasons, reappears. Such strains have been noted from time to time by ourselves as well as others, and the purpose of the present paper is to describe the results of guinea pig inoculations in which a number of strains taken directly from patients were carried on in series of considerable length with irregularity in the appearance of swelling. The experiments described deal with a strain isolated at Jilotzingo which is spoken of briefly as the "J" strain.

Appearance of Scrotal Swelling in Guinea Pigs Directly Inoculated from Patients

Table I shows that inoculations from three patients in the same epidemic, the blood of each injected into three guinea pigs, resulted in positive reactions in all three of the guinea pigs from Patient 1; in none of those inoculated from Patient 2 and in only one guinea pig inoculated from Patient 3. In only one animal of the four successful inoculations—successful as far as fever reaction is concerned—did scrotal swelling appear, but it is important to note that the typical

swelling in this case was produced directly as the result of inoculation with human blood.

The Frequency of Scrotal Swelling in Guinea Pigs Inoculated in Series with the "J" Strain

The observations recorded in Table II are important in that there was only an occasional scrotal reaction in the guinea pigs inoculated up to the sixth generation. Then, for eight generations, from the seventh to the fifteenth, the scrotal reaction was absent. After that it recurred in two guinea pigs of the sixteenth generation. It was

TABLE I

Patient	Guinea pig	Results
1	1	Fever, no swelling
	2	Fever, swelling
	3	Fever, no swelling
2	4	No fever or swelling
	5	No fever or swelling
	6	No fever or swelling
3	7	No fever or swelling
	8*	Fever
	9	No fever or swelling

* Guinea pig 8 was a female, the others being males.

In this experiment guinea pigs were carefully selected to avoid contamination with *Salmonella*, which frequently occurs in Mexico City.

lost again from the seventeenth to the twentieth generation, and again from the twenty-second to the twenty-third, but reappeared in the twenty-fourth generation.

Rickettsia were not found in any of the animals showing swelling until the twenty-fourth generation. This we are inclined to attribute to the fact that we had not yet learned that the rickettsia are difficult to find unless the examination is made quite early. At the height of the swelling—4 to 6 days after inoculation—the rickettsia are much more difficult to find than when the tunica is examined at the very first indication of reaction, or even before reaction has become manifest.

At any rate, the experiments recorded in the table show that after prolonged failure of scrotal swelling, the typical Neill-Mosser reaction with rickettsia can appear, a point that would suggest strongly that the rickettsia and the lesion in the tunica vaginalis are truly part of the typhus fever and not incidentally carried along.

Cross Immunity Experiments

We considered it of some importance also to determine whether a typhus fever without scrotal swelling in guinea pigs immunized to subsequent inoculation with a Mooser strain which produced swelling

TABLE II

No. of passage	Material injected	No. of guinea pigs with typhus fever	No. of guinea pigs with swelling	Rickettsia	No. of guinea pigs with no swelling
1st	Human blood	4	1	0	3
2nd	Guinea pig blood	5	0		5
3rd	Guinea pig blood	5	1	0	4
4th	Guinea pig blood	6	2	0	4
5th	Guinea pig blood	4	1	0	3
6th	Guinea pig blood	4	1 (slight)	0	3
7th to 15th	Guinea pig blood	24	0		24
16th	Guinea pig blood	3	2	0	1
17th to 20th	Guinea pig blood	10	0		10
21st	Guinea pig blood	2	1	0	1
22nd to 23rd	Guinea pig blood	4	0		4
24th	Guinea pig blood	2	1 (early castrated)	++++	1
25th	Guinea pig blood	2	0		2

with considerable regularity. Of thirty-three guinea pigs inoculated with the "J" strain in this series, only four showed scrotal swelling. Nine were unsuccessfully inoculated, showing neither fever nor swelling. Twenty, which showed typical fever curves but no swelling of the scrotum, were subsequently reinoculated with the typical Mooser strain and in no case developed either swelling or fever.

The nine unsuccessfully inoculated pigs were all reinoculated similarly and all of them developed both fever and swelling.

From these experiments it is clear that a typhus fever conveyed

by the "J" strain and recovered from without scrotal lesion protects against the scrotal lesion upon subsequent inoculation with the typical Mooser strain.

This would again indicate that the scrotal lesion is merely an incidental localization in some guinea pigs of the disease which is immunized against by a general typhus fever, even if in the course of the latter this lesion is absent.

TABLE III

Guinea pig	Passage No.	Fever	Swelling	Rickettsia
a	2nd	+	—	0
b	3rd	+	+	
c	4th to 15th	+	—	
d	16th	+	+	0
e	17th to 20th	+	—	0
f	21st	+	+ slight	
g	22nd to 24th	+	—	
h	25th	+	+	Very few extra-cellular

From Guinea pig "h" (25th passage) lice were then intrarectally inoculated with tunica by the Weigl method and after 10 days this material was injected into guinea pigs with the following results:

Guinea pig	Lice strain. Passage No.	Fever	Swelling	Rickettsia
i	1st	+	+	++++
j	2nd	+	+	++++
k	3rd to 5th	+	—	++++
l	6th	+	+	

The Influence upon the Ability of a Strain to Cause Scrotal Swelling after Passage through Lice

Table III again shows the "J" strain, carried in twenty-five generations with frequent absence of scrotal swelling for a number of generations, even after the twenty-fifth generation is passed through lice and reinoculated into guinea pigs. In the first two generations after passage through lice, typical swelling with abundance of rickettsia

occurred, then again for two generations swelling and rickettsia were absent, in spite of typical febrile reactions, to reappear in the sixth generation as markedly as they had been present before.

SUMMARY

The experiments recorded above have demonstrated the following points:

1. Scrotal swelling can appear in guinea pigs directly inoculated from a human case of Mexican typhus fever.

2. In certain strains of this disease, a number of generations of guinea pigs may show absolutely no scrotal swelling, which, however, may reappear in subsequent animals, suggesting—though not absolutely proving—that the scrotal swelling is an integral part of the disease and is not due to an incidental accompanying organism. If the latter were true, one would expect the organisms that caused the scrotal swelling to disappear during the negative generations.

3. A typhus fever sustained by a guinea pig without scrotal swelling protects against the swelling upon subsequent inoculation with a strain which produces this with considerable regularity.

4. Louse passage increases the capacity of a strain to produce the scrotal lesion, probably because of the considerable accumulation of rickettsia in the louse, but in the experiment noted, even after louse passage, two generations without swelling occurred, followed by reoccurrence of the swelling.

We believe that these observations, taken together, can be interpreted in favour of the likelihood that the swelling is a part of the disease and that the rickettsia-like organisms described by Mooser in the tunica vaginalis have etiological significance.

STUDIES ON INFLAMMATION

IV. FIXATION OF FOREIGN PROTEIN AT SITE OF INFLAMMATION

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In the previous papers of this series it was shown that trypan blue injected directly into an inflamed area was fixed *in situ* and failed to reach the tributary lymph nodes (1). When this dye was introduced into the circulating blood stream it rapidly entered the inflamed area and was fixed there. Subsequent studies showed that the rapid accumulation of dye in an inflamed area is the result of increased capillary permeability (2). These studies were then extended and it was found that colloidal iron or ferric chloride injected directly into an inflamed area was fixed *in situ* by the inflammatory process, and that ferric chloride injected intravenously rapidly entered inflamed cutaneous areas, where its presence was identified by both qualitative and quantitative determinations (3).

The earlier literature on the dissemination of substances from the site of injection has been reviewed in previous publications (1 and 2). Opie (4) showed that foreign protein injected into the skin of an actively immunized animal is fixed at the site of injection where the contact of antigen and antibody causes an acute inflammatory reaction (Arthus phenomenon). In view of this work on immunized animals and of the results obtained with trypan blue and iron, experiments were undertaken to determine whether a readily identified foreign protein such as horse serum injected into an area of inflammation caused by either bacteria or a sterile irritant would also be retained *in situ* by the inflammatory reaction.

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Penetration of Horse Serum into the Blood Stream after Its Injection into the Inflamed Peritoneal Cavity

Horse serum varying in quantity from 2 to 8 cc. was injected into the peritoneal cavity of normal rabbits and of rabbits that had previously been given an intraperitoneal injection of an inflammatory irritant. Sterile inflammation was induced by a mixture of 5 per cent of aleuronat and 3 per cent of starch in 0.5 per cent saline solution. This mixture was injected into the peritoneal cavity 24 to 48 hours prior to the injection of horse serum.

Samples of blood were removed from the heart at varying intervals after the intraperitoneal injection of horse serum. The blood was centrifugalized and the serum tested for horse serum by anti-horse-serum obtained from rabbits repeatedly injected with this foreign protein. The blood serum was diluted by the addition of 2 cc. of saline to 1 cc. of serum. Varying dilutions of blood serum in multiples of 3 were then made from this initial dilution. The final tests were performed by the addition to 0.7 cc. of saline of 0.1 cc. of the diluted blood serum and 0.2 cc. of anti-horse-serum.

Tables I, II, and III summarize all the experiments by giving the results of tests for presence of horse serum in the blood at varying intervals after intraperitoneal injection of the foreign protein. These tests serve as a rough measure of the concentration of the antigen in the blood serum. A few illustrative experiments follow.

Experiment 10.—Rabbit 17 was injected with 4 cc. of horse serum when an inflammatory reaction had been in progress for about 48 hours. Rabbit 8 served as control receiving 4 cc. of horse serum intraperitoneally. The results of tests for presence of horse serum in the blood 2 hours after its injection appear in Table I. The results of tests 6 hours and 24 hours later are shown in Tables II and III respectively. Following the introduction of horse serum into the inflamed peritoneal cavity its concentration in the blood is conspicuously less after 2 and 6 hours than that found after injection into the normal peritoneal cavity; but after 24 hours the horse serum in the blood of both animals has the same concentration.

In the following experiment samples of blood were removed from the heart about 6 hours after injection of horse serum into the normal and inflamed peritoneal cavity of rabbits.

Experiment 13.—4 cc. of horse serum were injected into the peritoneal cavity of Rabbit 20. Rabbit 23, in which peritonitis had been induced 24 hours previously, received the same amount of horse serum. 5½ hours later samples of blood were removed from the heart and tested for the presence of horse serum, as already described. The results appear in Table II.

TABLE II
Presence of Horse Serum in the Blood Stream 5 to 7 Hours after Intraperitoneal Injection

Experi- ment No.	Duration of inflam- mation (hours)	Animals with inflamed peritoneal cavity										Animals with normal peritoneal cavity									
		Dilutions								Dilutions											
		1:3	1:9	1:27	1:81	1:243	1:729	1:2187	1:3	1:9	1:27	1:81	1:243	1:729	1:2187						
13	30	++	+	Trace	0	0	0	0	++	++	+	+	Trace	0	0						
5	31	++	+	Trace	0	0	0	0	++	++	+	Trace	0	0	0						
12	33	Trace	0	0	0	0	0	0	++	+	Trace	0	0	0	0						
15	52	+	+	Trace	0	0	0	0	++	++	+	Trace	0	0	0						
11	54	++	+	Trace	0	0	0	0	++	+	Trace	Faint	0	0	0						
10	55	+++	+	Trace	0	0	0	0	++	+	+	Trace	0	0	0						

TABLE III
Presence of Horse Serum in the Blood Stream 24 to 30 Hours after Intraperitoneal Injection

Experi- ment No.	Animals with inflamed peritoneal cavity														Animals with normal peritoneal cavity													
	Duration of inflam- mation (hours)	Dilutions								Dilutions																		
		1:3	1:9	1:27	1:81	1:243	1:729	1:2187	1:3	1:9	1:27	1:81	1:243	1:729	1:2187													
4	49	+++	++	+	+	Trace	0	+++	++	+	+	Trace	0	0														
5	50	+++	+	+	Trace	0	0	+++	++	+	0	0	0	0														
8	50	+++	++	+	Trace	Trace	0	+++	++	+	+	Trace	0	0														
9	50	+++	++	+	Trace	0	0	+++	++	+	+	Trace	0	0														
10	70	+++	++	+	Trace	0	0	+++	++	+	Trace	0	0	0														
11	71	++	+	+	0	0	0	++	+	0	0	0	0	0														
6	78	0	0	0	0	0	0	+++	++	++	Trace	0	0	0														

From this experiment it is evident that 5 hours and 30 minutes after injection of horse serum into a peritoneal cavity that has been inflamed for 24 hours the penetration of the foreign protein into the blood stream is delayed.

A similar experiment in which the inflammatory process had been going on for 48 hours before the injection of foreign protein follows.

Experiment 15.—Rabbit 21 received 4 cc. of horse serum intraperitoneally; 5 hours later 3 cc. of blood were removed from the heart. Rabbit 24 was injected with the same amount of horse serum 47 hours after receiving 10 cc. of the aleuronat suspension intraperitoneally. 5 hours after the injection of horse serum 3 cc. of blood were withdrawn from the heart. Tests for the presence of horse serum in the blood of both of these rabbits yielded the results shown in Table II.

It is clear that there has been a definite retardation in the passage of the protein after its injection into an inflamed peritoneal cavity.

Samples of blood withdrawn from the heart 24 to 30 hours after injection of horse serum into both normal and inflamed peritoneal cavities showed in most instances no appreciable differences (Table III).

Experiment 4.—4 cc. of horse serum were injected into the peritoneal cavity of Rabbit 13, 24 hours after aleuronat had been injected into the cavity. 2 hours later this animal was bled from the heart. Rabbit 4 which served as control received intraperitoneally 4 cc. of horse serum and was bled 2 hours later. The results of this experiment appear in Table I. It is seen that after injection of protein into the inflamed peritoneal cavity its concentration in the circulating blood is distinctly lower than after injection into the normal cavity. A second sample of blood was removed 25 hours after the injection of horse serum.

The presence of horse serum in the blood stream 1 day after intraperitoneal injection of the protein is recorded in Table III. Although there was delay in the penetration of horse serum into the blood stream 2 and 6 hours after injection into an inflamed peritoneal cavity, the concentrations of horse serum in the blood of experimental and control animals are now equal.

The Retention of Horse Serum at the Site of Inflammation

In the above experiments it was shown that when horse serum is injected into an inflamed peritoneal cavity its passage into the blood

stream is delayed during several hours. To determine whether this delay is due to retention of the foreign protein in the inflamed peritoneal cavity a series of experiments was undertaken to measure directly changes in the quantity of horse serum at the site of inflammation.

10 cc. of 5 per cent aleuronat and 3 per cent starch in 0.5 per cent saline were injected into the peritoneal cavity of rabbits. 24 to 48 hours later 2 to 4 cc. of horse serum were injected into the inflamed peritoneal cavity. Each experiment was controlled by the injection of the same amount of horse serum into the peritoneal cavity of a normal rabbit. 2 to 5 hours later 10 cc. of saline were injected into both normal and inflamed peritoneal cavities. This was done in order to be certain of obtaining a sufficiently large sample of fluid for the tests. The rabbits were then immediately bled from the heart and the abdomen was opened under anesthesia with ether. The peritoneal fluid was tested for the presence of horse serum. A typical experiment follows.

Experiment 11.—Rabbit 9 received an injection of 2 cc. horse serum intraperitoneally. 2½ hours later 10 cc. of saline were injected into the peritoneal cavity. Immediately afterwards 4 cc. of blood were withdrawn from the heart. About 3 cc. of serous peritoneal fluid were obtained. The same technique was followed in Rabbit 18, which had, however, received an intraperitoneal injection of 10 cc. of aleuronat suspension 47 hours before. Tests for the presence of horse serum in the blood are given in Table I. Tests for the presence of horse serum at the site of inflammation are shown in Table IV.

Essentially the same results were obtained in the following experiment when the horse serum was injected 5 hours previous to the removal of the samples of blood and peritoneal fluid.

Experiment 15.—4 cc. of horse serum were injected into the normal peritoneal cavity of Rabbit 21; 5 hours later 10 cc. of saline were injected into the cavity and immediately afterwards 3 cc. of blood were withdrawn from the heart. About 1 cc. of serous fluid was then obtained from the peritoneal cavity. Rabbit 24 was given an injection of 10 cc. aleuronat suspension into the peritoneal cavity and 47 hours later 4 cc. of horse serum. 5 hours later samples of peritoneal fluid and blood were obtained as described for Rabbit 21. The tests for presence of horse serum at the site of inflammation are shown in Table IV, and in the blood stream in Table II.

It is evident from these experiments that there is a greater retention of horse serum *in situ* when the foreign protein is injected into an inflamed peritoneal cavity than when injected into a normal one. This observation is correlated with the delay in the penetration of the protein into the blood stream.

It has been seen (1) that when the inflammatory reaction was in-

TABLE IV
Retention of Horse Serum at Site of Inflammation

Experiment No.	Interval between injection of horse serum and tests for its presence (hours; minutes)	Total duration of inflammation (hours)	Animals with inflamed site previous to injection of horse serum								Animals with normal site previous to injection of horse serum									
			Dilutions										Dilutions							
			1:3	1:9	1:27	1:81	1:243	1:729	1:2187		1:3	1:9	1:27	1:81	1:243	1:729	1:2187			
14	2:30	26	+	++	+	+	Trace	0	0		+	Trace	0	0	0	0	0			
11	2:30	50	++	++	+	Trace	Trace	0	0		++	+	+	0	0	0	0			
15	5:00	52	++	++	+	+	+	Trace	0		++	+	+	Trace	0	0	0			
16*	24:00	42	+	++	+	Trace	Trace	0	0		+	Trace	0	0	0	0	0			
17*	24:00	48	++	+	Faint trace	0	0	0	0		+	0	0	0	0	0	0			

* Site of injection of horse serum and inflammation was located in skin.

duced in the peritoneal cavity of rabbits the fixation of trypan blue was less effective than when the subcutaneous tissue was the site of the experimental inflammation. For this reason horse serum was injected into an inflamed area on the skin of the abdomen.

About 0.2 cc. of a saline suspension of *Staphylococcus aureus* was injected intracutaneously. 24 hours later 0.15 cc. of horse serum was injected directly into the inflamed area, which was removed 24 hours later, weighed and cut into small pieces. These were thoroughly ground in a mortar with a volume of saline equal to 10 times the weight of the dermal area. The extract was centrifugalized at rapid speed for about 45 minutes. In the control experiment 0.15 cc. of horse serum was injected into the normal skin of a rabbit and the tissues were extracted in saline 24 hours later. The results of two such experiments are shown in Table IV.

Retention of the foreign protein is evident 24 hours after its injection into an area of cutaneous inflammation.

These experiments show that like trypan blue and iron, complex foreign proteins such as are found in horse serum when injected into an inflamed area are retained *in situ* by the inflammatory reaction and penetrate less rapidly into the circulating blood stream than when injected into normal tissue. Opie (4, 5), as has been pointed out, showed that when foreign protein is first injected into the skin of a normal animal it rapidly leaves the site of injection and enters the circulating blood. In an animal that has been immunized reinjection with the foreign protein produces an intense inflammatory reaction with necrosis at the point of entry. The foreign protein is fixed at the site of injection, and fails to enter the circulating blood. The results that the writer has obtained are in agreement with these observations and show that an inflammatory reaction (caused in Opie's experiments by the contact of antigen and antibody and in these experiments by a sterile irritant) arrests the protein at the point of entry.

The Accumulation of Horse Serum in Inflamed Cutaneous Areas

Having demonstrated that the penetration of horse serum into the blood stream is delayed at the site of inflammation the attempt was made to determine whether horse serum injected into the circulating blood stream would accumulate in inflamed areas to a greater extent

TABLE V
The Accumulation of Horse Serum in Inflamed Cutaneous Areas

Experiment No.	Inflamed skin								Normal skin							
	Dilutions								Dilutions							
	1:3	1:9	1:27	1:81	1:243	1:729	1:2187		1:3	1:9	1:27	1:81	1:243	1:729	1:2187	
21	+	+	Trace	Faint	0	0	0		Faint	0	0	0	0	0	0	0
22	++	+	Trace	trace	0	0	0		trace	0	0	0	0	0	0	0
23	+	Trace	0	0	0	0	0		Trace	0	0	0	0	0	0	0
24	+++	++	+	Trace	0	0	0		+	Trace	Faint	0	0	0	0	0
25	+	+	+	Trace	0	0	0		+	+	Trace	0	0	0	0	0
26	++	++	+	Trace	0	0	0		++	+	Trace	0	0	0	0	0
27	++	++	+	Trace	0	0	0		+	+	Trace	0	0	0	0	0
28	+++	++	+	Trace	0	0	0		+++	+	0	0	0	0	0	0
29	+++	++	+	0	0	0	0		+++	+	+	0	0	0	0	0
30	++	+	+	0	0	0	0		+	Trace	0	0	0	0	0	0

than in normal tissue, as had been observed with trypan blue and ferric chloride.

Areas of cutaneous inflammation were induced by the injection of about 0.2 cc. of a saline suspension of *Staphylococcus aureus* into the skin of the abdomen of rabbits. About 3 hours later 10 cc. of horse serum were injected intravenously. The animal was killed when the inflammation was of 5 to 6 hours duration. The inflamed areas were removed and weighed. They were then cut into thin strips and ground in a mortar with a volume of saline equal to twice the weight of the tissue. In earlier experiments the extracts were obtained by grinding with saline alone, but in subsequent experiments by means of sand and saline. As control areas, strips of normal skin from the abdomen of the same rabbit were removed and the foreign protein extracted by the same technique as with the inflamed areas. The extracts were centrifugalized at rapid speed for 45 minutes. After the top layer of fat was carefully removed, 1 cc. of the extract was drawn off with a pipette into a test tube containing 2 cc. of saline. Dilutions of the extract in multiples of 3 were then prepared from this first tube. In the final test 0.1 cc. of diluted extract was added to 0.2 cc. of anti-horse-serum and the mixture made up to 1 cc. with saline. The final dilutions were kept 1 hour in a water bath at 38°C. and then placed overnight on ice. Readings were made on the following morning. The results of all the experiments are shown in Table V.

In all the experiments there is consistently a greater concentration of the foreign protein in inflamed than in normal tissue. These results are therefore similar to those obtained with trypan blue and with ferric chloride. The accumulation of foreign protein in inflamed tissue is doubtless in part the result of the increased passage of fluid from the circulating blood stream, but observations recorded above indicate that its escape from the site of inflammation is retarded.

Some years ago Auer (6) showed that if the ear of a rabbit immunized with horse serum is painted with xylol, reinjection of the antigen causes in the affected ear an intense inflammatory reaction followed by necrosis. No such severe reaction was seen when xylol had been applied to the ear of a normal rabbit after a single injection of horse serum. In view of the observations on the accumulation of foreign protein in inflamed areas it is highly probable that the reaction in Auer's experiment is simply the result of an accumulation from the circulating blood stream of antigen and antibody in the inflamed ear of sensitized animals; the contact of antigen and antibody in the tissues

causing an acute inflammatory reaction and intensifying thus the mild reaction caused by xylol alone.

A few years ago Opie (personal communication) repeated the experiments of Auer with crystalline egg albumin and instead of applying xylol as an inflammatory irritant, he dipped the ear of the experimental animal into water at 50°C. for 5 minutes. On the following day crystalline egg albumin was injected into the peritoneal cavity of the animal. Several days later necrosis of the inflamed ear was observed in many instances.

Experiment 31.—Rabbit 30 received 5 injections of 1 cc. horse serum administered subcutaneously at 5 to 6 day intervals. 19 days after the last injection 10 cc. of horse serum were injected into the peritoneal cavity. 1 hour later an ear of this animal was rubbed lightly for a minute with a pledget of cotton soaked in xylol. 2 days later the ear showed only a very mild reaction. It was then placed in water at 54°C. for 5 minutes. It immediately flushed. 10 cc. of horse serum were then reinjected into the peritoneal cavity. 24 hours later the ear was very much congested and edematous, revealing a pronounced inflammatory reaction.

The animal was etherized and bled to death. The inflamed and the normal ear were removed. The weight of the inflamed ear was 13.5 gm. and of the normal ear 5 gm. Each ear was then cut into thin strips and ground separately in a mortar with a volume of saline equal to its own weight. The extracts were then centrifugalized at rapid speed for 40 minutes and the supernatant fluid tested for the presence of horse serum. As control to this experiment, Rabbit 31, which had not been sensitized to the foreign protein, was treated in the same manner as Rabbit 30. The results of this experiment are shown in the following table:

Dilutions of skin extract	Immunized Rabbit 30		Normal Rabbit 31	
	Normal ear	Inflamed ear	Normal ear	Inflamed ear
1:3	++	+++	Trace	++
1:9	Trace	++	Faint trace	+
1:27	0	+	0	Faint trace
1:81	0	+	0	0
1:243	0	0	0	0
1:729	0	0	0	0
1:2187	0	0	0	0

When xylol alone was used as the inflammatory irritant, essentially the same results were obtained.

Experiment 32.—Rabbit 32 received 5 injections of 1 cc. horse serum administered subcutaneously at 3 to 5 day intervals. 5 days after the last injection 10 cc

of horse serum were injected into the peritoneal cavity. 1½ hours later the right ear was rubbed gently with 2 cc. of xylol for about 2 minutes. The application of xylol was repeated twice in the next 2 hours at 1-hour intervals. 24 hours later the ear was mildly edematous and congested, with a few blisters.

The animal was killed under ether and both ears were removed. The weight of the inflamed ear was 3.7 gm. and of the normal ear 2.2 gm. Each ear was cut into thin strips and ground separately in a mortar with a volume of saline equal to twice its own weight. The extracts were then centrifugalized at rapid speed for 1½ hours. After being filtered, the supernatant fluid was tested for the presence of horse serum. The samples containing the extract in a dilution of 1 to 3 were not read owing to slight cloudiness in the control tubes in which anti-horse-serum was absent. As control to this experiment, Rabbit 33, which had not been sensitized to the foreign protein, was treated in the same manner as Rabbit 32. The results of this experiment are shown in the following table:

Dilutions of skin extract	Immunized Rabbit 32		Normal Rabbit 33	
	Normal ear	Inflamed ear	Normal ear	Inflamed ear
1:3			+	++
1:9	+	++	+	++
1:27	Faint trace	+	0	+
1:81	0	Trace	0	Faint trace
1:243	0	0	0	0
1:729	0	0	0	0
1:2187	0	0	0	0

These experiments show that horse serum accumulates from the circulating blood in the inflamed ear in greater concentration than in the normal ear. The severe inflammatory reaction in the ears of the sensitized animals in the experiments of Auer, when the same antigen was reinjected, was doubtless due to the accumulation and contact in the inflamed tissues of antigen and antibody from the circulating blood.

It is possible that the phenomenon of focal reaction in tuberculosis can be explained by the observations reported in this paper. When tuberculin is introduced into the blood stream of an animal with a tuberculous lesion, an intense inflammatory reaction may develop in the lesion. The mechanism of this focal reaction in tuberculosis is not understood. It is conceivable in view of the above experiments with horse serum that in a like manner tuberculo-protein may accumulate

from the blood stream in the inflamed tuberculous lesion and by its presence there induce a local inflammatory reaction.

CONCLUSIONS

Foreign protein such as horse serum injected into an inflamed peritoneal cavity penetrates into the blood stream less rapidly than when introduced into the normal cavity.

Foreign protein injected into a cutaneous inflammatory area is held *in situ* for a longer period than when injected into an inflamed peritoneal cavity.

Foreign protein introduced into the circulating blood stream accumulates in an inflamed area, where it is found in greater concentration than in normal tissue.

Accumulation of foreign protein at the site of inflammation explains the phenomenon of local anaphylaxis described by Auer in rabbits sensitized to horse serum. The antigen accumulating in the inflamed ear reacts with antibody, intensifies a pre-existing inflammatory reaction and produces necrosis of the ear.

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SUSCEPTIBILITY OF RABBITS TO INFECTION BY THE INHALATION OF TYPE II PNEUMOCOCCI

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In preceding papers (1) it has been shown that following inhalation, virulent Type I pneumococci generally disappear from the lungs of rabbits within a few hours, only to reappear later in certain instances in which a general septicemia occurs. It has further been shown that some animals may recover from a Type I pneumococcus septicemia. Rabbits are, nevertheless, very susceptible to infection by Type I pneumococci. In order to determine how they would react to less virulent strains of pneumococci, Type II pneumococcus was selected. Three different strains of Type II were used. One strain was converted into the "R" form, by repeated transfers in Type II anti-pneumococcus serum. The changes from the "S" to the "R" form are accompanied by loss of capsule, type specificity, and virulence for mice. This strain is designated *Pneumococcus IIR* and is avirulent for rabbits. The original *Pneumococcus* Type II strain is designated *Pneumococcus* Type II (SAv). An intraperitoneal injection of 0.1 cc. of a broth culture of this stock strain is necessary to kill rabbits. It was rendered rabbit virulent by rapid passage. This virulent strain, called *Pneumococcus* II (Sv), regularly kills rabbits following intraperitoneal injections of 0.000,01 cc.

In the present paper are reported (1) the distribution of pneumococci in the organs of rabbits following exposures to repeated inhalations of the above mentioned strains, (2) the occurrence of positive blood cultures, and (3) the mortality of the rabbits exposed to each of the above mentioned strains.

Method

The rabbits were placed in a large spray chamber similar to that already described (2) and exposed to a spray of pneumococci. 150 cc. of an 18 hour broth

culture was used for each spraying. The animals were exposed at 10 day intervals. Before each spraying, a sample of blood was obtained by bleeding the animal from the ear vein. At daily intervals after the first spraying, blood cultures were taken from a few of the rabbits. In the case of the animals which were killed and autopsied, broth cultures were made from both sides of the heart, and small pieces of the periphery of both lungs, kidney, liver, and spleen were placed directly in tubes of broth. Heart's blood cultures alone were made from the rabbits which died. All cultures were plated on blood agar for further identification. The animals that were killed were first chloroformed, immersed in a solution of lysol, and autopsied with sterile instruments.

	Liver		Spleen		Kidney		Right lung		Left lung		Right heart		Left heart	
	+	-	+	-	+	-	+	-	+	-	+	-	+	-
Hrs.														
1														
2														
3														
4														
5														
6														
Days														
1														
2														
3														
4														
5														

TEXT-FIG. 1. Distribution of IIR pneumococci in organs following spraying. The black squares indicate organs in which cultures showed pneumococci to be present, the cross-hatched squares represent organs in which cultures remained sterile. "c" = contamination.

Distribution of Pneumococci IIR in the Organs

In Text-fig. 1 is shown the distribution of IIR pneumococci in the organs of 44 sprayed rabbits which were killed at intervals after spraying. One to 6 hours after spraying pneumococci were only recovered from the periphery of the lungs in less than half of the animals sacrificed within the first 3 hours. Pneumococci were never recovered in this group from the liver, spleen or kidney. The heart's

blood cultures from these animals remained sterile. Of the remaining 20 rabbits killed from 1 to 5 days following exposure, pneumococci were only recovered from 2 rabbits killed on the 4th day. In one of these rabbits pneumococci were recovered from the liver, kidney and spleen and in another only from the kidney. This experiment shows how readily rabbits destroy the non-virulent R-forms of pneumococcus. These organisms were never recovered from the blood, rapidly disappeared from the lungs, and were rarely recovered from other viscera.

Daily blood cultures were taken from 7 rabbits following their first exposure to a spray of *Pneumococcus* IIR. During the 10 days period of observation in no instance were pneumococci recovered from the blood.

In all, 17 rabbits were exposed to from 3 to 10 inhalations of avirulent Type IIR pneumococci. Eight rabbits completed the course of 10 exposures. No rabbit became infected.

Distribution of Pneumococci Type II (SAv) in the Organs

In Text-fig. 2 is shown the distribution of the slightly virulent *Pneumococci* Type II (SAv) in the organs of 44 sprayed rabbits which were killed at intervals after spraying. It will be seen that pneumococci were recovered from the lungs of 3 of the 4 rabbits killed in 1 hour and that although the heart's blood cultures remained sterile, in one instance pneumococci were demonstrated in the culture from the spleen. After the 1st hour, however, the pneumococci were only recovered from the lungs in 3 instances. Of the remaining 20 rabbits killed from 1 to 5 days following exposure, 1, or 5 per cent, showed a general pneumococcus septicemia on the 2nd day. Although in the other rabbits pneumococci were not recovered from the lungs, in 2 instances the liver and kidney contained organisms and the spleen was positive in 1 rabbit. From the left heart's blood of one of the rabbits killed on the 5th day a positive culture was obtained but the culture of the right heart and spleen of this animal remained sterile. This experiment shows that a few rabbits may develop a generalised septicemia following inhalation of the slightly virulent *Pneumococcus* Type II (SAv).

	Liver		Spleen		Kidney		Right lung		Left lung		Right heart		Left heart	
	+	-	+	-	+	-	+	-	+	-	+	-	+	-
Hrs														
1														
2														
3														
4														
5														
6														
Days														
1														
2														
3														
4														
5														

TEXT-FIG. 3. Distribution of Type II (Sv) pneumococci in organs following spraying. The black squares indicate organs in which cultures showed pneumococci to be present, the cross-hatched squares represent organs in which cultures remained sterile. "c" = contamination.

TABLE II

Days on Which Positive Blood Cultures Were Obtained Following a Single Exposure to a Pneumococcus II (Sv)

Rabbit No.	Days										Course
	1	2	3	4	5	6	7	8	9	10	
1	-	-	-	-	c	+	+				Died 8th day
2	-	c	-	-	-	-	-	-	-	-	Survived
3	c	-	-	-	-	-	-	-	-	-	"
4	-	-	-	-	-	-	-	-	+	-	"
5	-	-	-	-	-	-	-	c	-	-	"
6	+	+	+								Died 4th day
7	-	-	-	-	-	-	-	-	-	-	Survived
8	-	c	+	c	-	-	-	-	-	-	"
9	-	-	-	-	-	-	-	-	-	-	"
10	+	c	-	-	-	-	-	-	-	-	"
11	-	+	+	+	-	-	-	-	-	-	"

+ indicates a positive culture of pneumococcus; - = a sterile culture; and "c" = a contaminated culture.

As is seen from Table II, of the 6 rabbits which developed septicemia 2 or 18 per cent continued to have a positive blood culture until death. The other 4 rabbits had merely a transient septicemia and survived. In some instances, the septicemia could be detected during the first 24 hours, but in others the organisms were first recovered as late as the 6th or 9th day.

TABLE III

Fate of Rabbits after One to Ten Exposures to Inhalation of Rabbit Virulent Type II Pneumococcus*

No. of times exposed to virulent pneumococci	No. of rabbits exposed	No. dying	Pneumococcus recovered from heart's blood	Pneumococcus not recovered from heart's blood
1	50	14	12	2
2	36	1	—	1
3	34	2	1	1
4	30	1	—	1
5	26	2	1	1
6	24	2	1	1
7	21	—	—	—
8	17	1	—	1
9	17	—	—	—
10	16	1	1	—

* The discrepancy between the total numbers and the sum of the numbers in the individual groups is due to the fact that certain of the animals that survived were used for intraperitoneal tests or for other experiments.

Mortality of Rabbits Following Exposure to Inhalation of Pneumococcus Type II (Sv)

Table III shows the number of normal rabbits which succumbed during the course of spraying with rabbit virulent *Pneumococcus* II (Sv). From this table it is seen that out of 50 rabbits 15 or 28 per cent died following the first exposure. *Pneumococci* were recovered from the heart's blood in 12 instances. Out of the total of 24 rabbits which died, in 16 instances the organisms were recovered from the heart's blood, and the cultures remained sterile in the other 8. Although care was exercised to open the spray boxes promptly 1 hour after spraying, these animals probably died of suffocation. This table further shows that the greatest number of deaths occur after the first exposure. It is

difficult to explain, however, how some rabbits which have successfully survived 5, or even 9, exposures finally become infected.

The interval elapsing between the time of spraying and death from pneumococcus septicemia is shown in Table IV. From this it is seen that there is great irregularity in the time of death following the initial exposure. In fact 4 rabbits only succumbed on the 10th day. It is interesting to observe that in rabbits which had survived the first exposure, infection was delayed and death occurred from the 7th to 10th day after spraying.

In no instance was there any gross evidence of pneumonia, although serous and serofibrinous pleurisy and serofibrinous pericarditis were common.

TABLE IV

Number of Days Elapsing between Spraying and Death of Rabbits from Pneumococcus Type II (Sv) Septicemia

	Days									
	1	2	3	4	5	6	7	8	9	10
Spray I.....	—	1	2	2	—	1	1	1	—	4
“ III.....	—	—	—	—	—	—	—	—	1	—
“ V.....	—	—	—	—	—	—	1	—	—	—
“ VI.....	—	—	—	—	—	—	1	—	—	—
“ X.....	—	—	—	—	—	—	—	—	1	—

DISCUSSION

From the foregoing experiments it appears that the susceptibility of rabbits to infection following inhalation of the same type of pneumococcus is in direct proportion to the virulence of the strain used.

The non-virulent IIR pneumococci may be recovered from the periphery of the lungs for a period of 3 hours after spraying. After this time they are only occasionally recovered from any part of the body. The slightly virulent Type II (SAv) and virulent Type II (Sv) may likewise be recovered from the lungs for a few hours following exposure. But in the case of the more virulent strains, there appears to be an almost immediate general dissemination of the organisms throughout the body, as is evidenced by the recovery of pneumococci from the

spleen. The organisms then disappear from the blood only to reappear after 48 hours. This reappearance is often in the form of a septicemia. The same phenomenon was noticed in the case of rabbits sprayed with virulent Type I pneumococci. It is unfortunate that the subsequent penetration of the inhaled pneumococci into the pulmonary tissues cannot be followed. But the great number of other organisms which normally inhabit the bronchi of rabbits, as Jones (3) has shown, renders cultural studies of the lungs difficult. Histological detection of pneumococci in the tissues is also unreliable when the organisms are present only in small numbers.

In all probability the majority of the organisms are destroyed either where they are first implanted or deeper in the lung tissue by the endothelial leucocytes or by poly-morphonuclear leucocytes. In any case, a few organisms occasionally filter through into the blood stream. Gaskell (4) believes that invasion of the blood stream by pneumococci probably always takes place in the early stages of an air-borne infection of the lung. The actual invasion of the blood stream must be much more common than is generally supposed. Not only are there probably few organisms free at any one time in the circulating blood but these few bacteria may even be within leucocytes and not in reality multiplying in the blood.

The occasional organisms which reach the blood are probably rapidly filtered out of the blood stream by the organs and locally destroyed. It is evident, however, that following the apparent disappearance of the pneumococci from the periphery of the lungs, they again appear, this time in the blood, and as a result may be recovered from all the organs. The point of this reinvasion is as yet uncertain.

The results of the blood cultures taken following the initial spraying parallel the distribution of pneumococci in the rabbits which were sacrificed. The cultures from the rabbits sprayed with avirulent degenerated non-virulent "R" forms pneumococci remained sterile; an occasional positive culture was obtained from the animals exposed to the slightly virulent Type II (SAv) organisms but no animals succumbed. Only in the case of the rabbits exposed to inhalations of rabbit virulent Type II (Sv) pneumococci did the more susceptible rabbits die of an overwhelming septicemia. But some rabbits were even able to overcome the septicemia following exposure to this rabbit virulent strain.

Following the initial spraying with a highly rabbit virulent Type II (Sv) pneumococcus the majority of susceptible rabbits died of an overwhelming septicemia without any attempt on the part of the body to localize the infection.

SUMMARY

1. The susceptibility of rabbits to inhalations of pneumococci varies in direct proportion to the virulence of the organism for rabbits.

2. When rabbits are exposed to a pneumococcus spray, irrespective of the virulence of the organism, the bacteria readily penetrate into the lower respiratory tract.

3. When rabbits are exposed to a spray of avirulent degenerated "R" form of pneumococcus, septicemia does not occur and pneumococci are seldom recovered from the liver, kidney or spleen.

4. When rabbits are exposed to a spray of slightly virulent Pneumococcus Type II (SAv) a non-fatal septicemia may occur and pneumococci may be recovered from the liver, kidney and spleen.

5. When rabbits are exposed to a spray of virulent Type II (Sv) pneumococci, septicemia may occur which in certain instances terminates fatally. Pneumococci may also be recovered from the liver, kidney and spleen.

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DEVELOPMENT OF AGGLUTININS AND PROTECTIVE ANTIBODIES IN RABBITS, AFTER INHALATION OF TYPE II PNEUMOCOCCI

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In preceding papers (1) it has been shown that mice and rabbits may acquire a high degree of immunity, following repeated inhalations of living pneumococci. In this work it was shown that the strain of Type I pneumococcus used produced a high mortality among the exposed rabbits and that many of the survivors have agglutinins and protective antibodies in their serum. In the present study it seemed interesting to determine the immunity response to less virulent strains. For this purpose a Type II pneumococcus was used. Pneumococcus Type II, however, is relatively avirulent for rabbits. One strain was transformed into the "R" form by repeated transfers in antipneumococcus Type II serum. The changes to the "R" form are accompanied by loss of capsule, type specificity, and virulence. This strain is referred to as *Pneumococcus IIR*. The stock culture of *Pneumococcus* Type II would cause a fatal septicemia in rabbits when injected intraperitoneally in doses of at least 0.1 cc. This strain is called *Pneumococcus* Type II (SAv). By repeated animal passages the virulence of this strain was raised so that an intraperitoneal injection of 0.000,01 cc. of broth culture would regularly kill rabbits. This virulent culture is designated as *Pneumococcus* Type II (Sv).

It has already been shown that rabbits are only susceptible to fatal infection by inhalations of virulent Type II pneumococci (Sv) (2). Although pneumococcus septicemia may be caused in an occasional rabbit following inhalation of the slightly virulent *Pneumococcus* Type II, death does not ensue. In the present paper is reported the development of (1) agglutinins and (2) protective antibodies in the blood serum of rabbits following repeated inhalations of aviru-

lent IIR, slightly virulent Type II (SAv), and virulent Type II (Sv) pneumococci.

Method

Rabbits were placed in a large spray chamber similar to that already described (3) and exposed to a spray of pneumococci. 50 cc. of an 8 hour broth culture were used for each spraying. The animals were exposed at 10 day intervals. Before each spraying, a sample of blood was obtained from the ear vein of each animal. None of the normal rabbit sera contained agglutinins nor did the normal sera protect mice against intraperitoneal injections of 0.000,001 cc. of Type II pneumococcus.

10 days after each exposure the presence of agglutinins in the serum of exposed animals was determined by a modified thread reaction. To 1 cc. of rabbit serum diluted in normal salt solution was added 0.2 cc. of an actively growing broth culture of *Pneumococcus* Type II. The tubes were incubated for 2 hours in the water bath at 37°C., placed in the ice box overnight, and the reactions read the next morning. Agglutinins were recorded as present in the serum only when the reactions were positive in a dilution of at least 1:10.

The presence of protective antibodies in the blood of the sprayed rabbits was determined by the ability of 0.2 cc. of serum to protect white mice against intraperitoneal injection of 0.001 cc. of *Pneumococcus* Type II culture, of which 0.000,001 cc. killed a normal mouse within 48 hours. The rabbit serum and culture were administered simultaneously.

Pneumococcus IIR

Of the 17 rabbits which were sprayed from 1 to 10 times with "R" forms derived from Type II pneumococcus none died from pneumococcus septicemia. Not even the 8 rabbits which were exposed 10 successive times developed any demonstrable agglutinins for Type II pneumococcus.

Neither did the sera of any of these rabbits protect mice against intraperitoneal injections of 0.001 cc. of a virulent culture of *Pneumococcus* Type II. As is seen from Table I the sera of these 5 animals, however, showed evidence of slight protection as demonstrated by the presence of a low degree of protective antibodies. Even the slight amount of protection afforded by these sera is not constant.

Pneumococcus Type II (SAv)

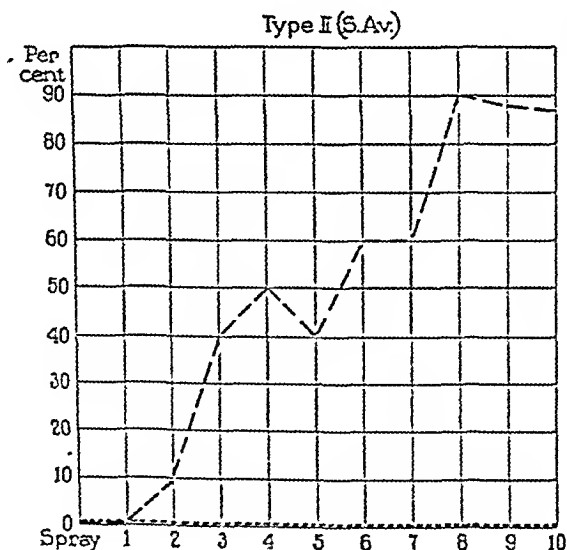
Of 11 rabbits sprayed from 1 to 10 times with the slightly virulent *Pneumococcus* Type II (SAv), none died from pneumococcus septi-

cemia. Not even the 8 rabbits which had completed the whole course of 10 exposures developed any demonstrable agglutinins for Type II pneumococcus.

TABLE I

First Appearance and Degree of Protection Following Repeated Exposure to Pneumococcus IIR

Following exposure.....	3	4	5	6	7	8	9	10
Rabbit 1.....	10^{-6}	—	10^{-6}	—	10^{-6}	10^{-6}	10^{-5}	—
" 2.....	—	—	—	—	—	—	—	10^{-5}
" 3.....	—	—	—	10^{-5}	10^{-5}	10^{-6}	10^{-5}	10^{-4}
" 4.....	—	—	—	—	10^{-6}	10^{-6}	10^{-6}	—
" 5.....	—	—	—	—	—	—	10^{-6}	—



TEXT-FIG. 1. Comparison of presence of protective antibodies and agglutinins in rabbits following repeated inhalations of slightly virulent Type II (SAv) pneumococci.

----- per cent of rabbits showing agglutinins in serum following spraying.
 — — — — per cent of rabbits showing protective antibodies in their serum.

Protective Antibodies

In Table II is shown the number of rabbits which were repeatedly sprayed from 1 to 10 times with *Pneumococcus* Type II (SAv) and the number thus exposed of which the serum subsequently protected mice against intraperitoneal injection of 0.001 cc. of a virulent culture of Type II pneumococcus. From this table it is seen that protective antibodies were demonstrable in the sera of 1 of the rabbits after the 1st spray. Following each successive spraying, the percentage of

TABLE II

Number of Rabbits Exposed to Pneumococcus II (SAv) and Number Yielding Serum Which Protected Mice against 0.001 Cc. of Pneumococcus Type II

No. of exposures.....	1	2	3	4	5	6	7	8	9	10
No. of rabbits.....	11	10	10	10	10	10	10	10	9	8
" " " whose serum protected.	—	1	4	5	4	6	6	9	8	7
Per cent of rabbits whose serum protected.....	—	10	40	50	40	60	60	90	88	87

TABLE III

First Appearance of Protective Antibodies in the Serum of Rabbits Exposed to Inhalation of Pneumococci Type II (SAv)

No. of spray.....	1	2	3	4	5	6	7	8	9	10
No. of rabbits showing protective antibodies.....	—	1	3	1	1	1	1	1	—	—

rabbits showing protection steadily increased, until after the 8th spraying the serum of 90 per cent of the animals conferred passive protection on mice against at least 1000 lethal doses of virulent cultures. The development of protective antibodies is graphically shown in Text-fig. 1. The exposure following which the rabbit sera first protected mice is shown in Table III. From this it is seen that with each successive spray the number of rabbits in the sera of which protective antibodies were demonstrable progressively increased. Certain rabbits showed protective antibodies only after the 7th or 8th exposure.

Pneumococcus II (Sv)

Of 50 rabbits sprayed from 1 to 10 times with the virulent strain of Type II (Sv) pneumococcus, 12 or 24 per cent died of pneumococcus septicemia after the first exposure. As the more susceptible animals succumbed early, serum was obtained only from rabbits with the greatest natural resistance, individuals which had also acquired a certain degree of immunity, as the result of repeated exposures to live pneumococci.

TABLE IV

Relation of Development of Agglutinins to the Number of Exposures to Rabbit Virulent Pneumococcus II (Sv)

No. of exposures.....	1	2	3	4	5	6	7	8	9	10
No. of rabbits.....	38	34	32	29	23	22	21	17	16	15
" " " showing agglutinins....	3	3	2	2	1	2	2	2	2	2
Per cent of rabbits showing agglutinins.....	7	8	6	6	4	9	9	11	12	13

TABLE V

First Appearance and Titre of Agglutinins Following Repeated Inhalations of Rabbit Virulent Pneumococcus Type II (Sv)

Following exposure.....	1	2	3	4	5	6	7	8	9	10
Rabbit 1.....	1-20	—	—	—	—	—	—	—	—	—
" 2.....	1-10	1-10	—	1-10	—	1-10	1-10	1-20	1-40	1-40
" 3.....	1-10	1-50	1-200*	—	—	—	—	—	—	—
" 4.....	—	1-20	1-20	1-50	1-40	1-10	1-10	1-10	1-10	1-10

—, no agglutinins demonstrable in serum.

* Accidental death of rabbit following 3rd exposure.

In Table IV is shown the number of rabbits which were sprayed from 1 to 10 times whose serum showed agglutinins in dilutions of at least 1 to 10. From Table IV it is seen that 3 or 7 per cent of the 38 rabbits which survived the first exposure developed demonstrable agglutinins.

Only 4 rabbits developed agglutinins. In Table V are shown the exposures after which agglutinins first appeared and the titre of agglu-

tinins following each subsequent spraying. Three rabbits showed agglutinins after the first exposure. Following the initial appearance of the agglutinins there is a tendency for the titre to rise after subsequent exposures. But in one instance the agglutinins disappeared, and in another became less marked.

Protective Antibodies

In Table VI are shown the number of rabbits that were sprayed from 1 to 10 times with rabbit virulent *Pneumococcus* II (Sv) and the num-

TABLE VI

Number of Rabbits Exposed to Rabbit Virulent Pneumococcus Type II (Sv) and Number That Developed Protective Antibodies in Their Serum

No. of exposures.....	1	2	3	4	5	6	7	8	9	10
No. of rabbits.....	38	34	32	29	23	22	21	17	16	15
“ “ “ whose serum protected.	6	7	7	7	4	4	4	6	6	8
Per cent of rabbits whose serum protected.....	10	20	21	24	17	18	29	35	37	53

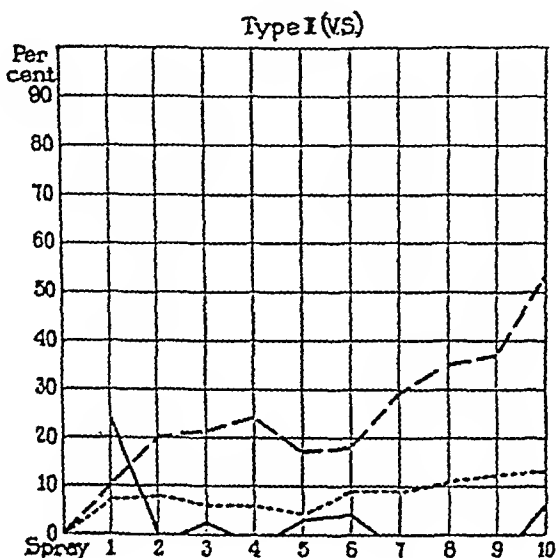
TABLE VII

First Appearance of Protective Antibodies

No. of spray.....	1	2	3	4	5	6	7	8	9	10
No. of rabbits first showing protective antibodies.....	6	1	—	1	—	—	—	1	1	3

ber thus exposed the serum of which subsequently protected mice against intraperitoneal injections of 0.001 cc. of a virulent culture of Type II (Sv) pneumococcus. From this table it is seen that protective antibodies were demonstrable in the sera of 4 or 10 per cent of the rabbits after the 1st spray. Following each successive spraying the number of rabbits yielding serum which conferred passive protection increased, until after the 10th spraying the serum of 8 or 53 per cent of the animals conferred passive protection on mice against at least 1000 lethal doses of virulent culture. The spray following which the sera first protected mice is shown in Table VII.

From Table VII it is seen that after the 1st exposure the sera of 6 rabbits showed protective antibodies. After successive sprays an occasional rabbit would first show protective antibodies. Protective substances were first demonstrated in the sera of 3 rabbits only after the 10th exposure.



TEXT-FIG. 2. Comparison of mortality and presence of protective antibodies and agglutinins in rabbits following repeated inhalations of virulent Type II (Sv) pneumococci.

———— per cent of rabbits dying with pneumococcus septicemia following successive sprayings.

----- per cent of rabbits showing agglutinins in serum following spraying.

- - - - per cent of rabbits showing protective antibodies in their serum.

Correlation of Agglutinins and Protective Antibodies

It is difficult to compare the relative titre of the sera at any one time because of the difference in the standards used. Whereas agglutinins were recorded as positive if present in serum concentration of 1:10, protective antibodies were noted only if they were present in concentrations sufficient to protect mice against a 0.001 cc. of virulent pneumococcus culture. If smaller doses of pneumococcus had been used, the

presence of protective antibodies would undoubtedly have been detected earlier and the incidence of their occurrence would have been more frequent. However, when these antibodies were demonstrable under the conditions of this experiment, their presence was evidence of the capacity of the serum to confer a high degree of passive immunity on mice.

In 3 instances both agglutinins and protective antibodies occurred after the same spray; in another rabbit, yielding serum that contained agglutinins, protective antibodies appeared after the next exposure. In 9 instances protective antibodies occurred without demonstrable agglutinins in the serum and the sera of 7 rabbits, even after 10 exposures, contained no agglutinins or protective antibodies even sufficient to protect mice against 0.000,001 of a virulent Type II pneumococcus.

The relative occurrence of agglutinins and of protective antibodies in the serums of rabbits following inhalations of rabbit virulent strain of Type II (Sv) pneumococcus is graphically shown in Text-fig. 2. In this figure the mortality curve of rabbits dying from pneumococcus septicemia is also given. It is seen that after the 1st exposure 24 per cent of the rabbits died from pneumococcus septicemia. An occasional rabbit died following subsequent exposures. The sera of the rabbits dying after the 3rd, 5th and 6th exposures showed neither agglutinins nor protective antibodies. The serum of the rabbit dying after the 10th spray, however, protected mice against 0.01 cc. of pneumococcus culture. It is unfortunate that it is not known if the pneumococcus recovered from this rabbit was the original type specific organism used for spraying or a degenerated "R" form. The number of rabbits that developed agglutinins was never great, nor was the titre high. The proportion of rabbits showing agglutinins rose only slightly following successive exposures. On the other hand, there was a distinct tendency for the proportion of rabbits to develop protective antibodies with each successive spray. Following the 10th exposure 53 per cent of the total number of survivals showed the presence of these immune substances in their serum.

DISCUSSION

Although too few rabbits were used to give as definite results as after spraying with Type I pneumococcus, some definite results are

indicated. It appears that the immunological reactions of rabbits following inhalations of the same type of pneumococci vary in direct proportion to the virulence of the organism. In the case of exposure to a non-virulent degenerated "R" strain derived from Type II pneumococcus no type specific antibody response was elicited. If rabbits are allowed to inhale slightly virulent Type II (SAv) pneumococcus, none succumb to infection, although a certain number develop a pneumococcus septicemia. As the result of successive spraying a large proportion of the animals developed protective antibodies in their serum but no agglutinins. Following the initial exposure to a rabbit-virulent Pneumococcus Type II (Sv) the greatest number of rabbits succumbed to a fatal septicemia. Certain rabbits developed a high degree of immunity as evidenced by the presence of agglutinin and protective antibodies in their serum. Why a larger proportion of rabbits exposed to the slightly virulent Pneumococcus Type II (SAv) developed protective antibodies than those sprayed with the virulent Pneumococcus Type II (Sv) is difficult to explain. The reaction of the rabbits to inhalations of the virulent Type II (Sv) culture parallels the results obtained when rabbits were sprayed with a rabbit-virulent Type I pneumococcus.

From the foregoing experiments it is certain that following inhalation of living virulent pneumococci of Type I or II rabbits develop a high degree of immunity. This immunity is probably induced by a few organisms penetrating the respiratory epithelium and entering into the body tissues. It has already been shown that rabbits may recover from a transient pneumococcus septicemia following inhalation of pneumococci. The great variations both in the time of first appearance and in the final titre of immune bodies are difficult to explain.

Among the factors which cannot be experimentally controlled are: first, the number of organisms which come to lodge within the respiratory tract following exposure to a bacterial spray; second, the number which are able to invade the tissues after implantation; and third, the final disposition of these bacteria in the animal body. In certain instances the multiplication of the invading organisms goes on unchecked until the death of the animal. In others a transient carrier state may occur with subsequent immunity responses. It is certainly significant that whereas the curve of incidence of protective antibodies

steadily increases, the percentage of rabbits showing agglutinins does not materially change after the 5th spraying.

CONCLUSIONS

1. Following repeated inhalations of the degenerated non-virulent "R" forms of Type II pneumococcus, no type specific antibodies can be demonstrated in the serum of rabbits.
2. Following repeated inhalations of slightly virulent Type II (SAv) pneumococci, only protective antibodies can be demonstrated in the serum of rabbits.
3. Following repeated inhalations of virulent Type II (Sv) pneumococci, agglutinins and protective antibodies can be demonstrated in the serum of rabbits.
4. Following repeated exposures of rabbits to inhalation of pneumococci, the type specific response, evidenced by type specific protective antibodies and agglutinins, varies in direct proportion to the virulence of the culture used.

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A TYPE SPECIFIC SUBSTANCE DISTINCT FROM THE SPECIFIC CARBOHYDRATE IN PNEUMOCOCCUS TYPE I

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The facts first determined by Avery and Heidelberger (1) and Zinsser and Tamiya (2) concerning the species specific nucleoprotein fraction of the pneumococcus as well as the type specific carbohydrate substance are at present so widely known and generally recognized that their recapitulation here would be superfluous. Aside from these fractions, other workers have described antigenic substances obtained by various chemical manipulations. Perlzweig and Steffen (3) in 1923 reported the finding of a material derived from the pneumococcus which was capable of active immunization upon injection into rabbits but which survived digestion with trypsin, was non-lipoidal, and gave Millon, xanthoproteic and ninhydrin reactions. Perlzweig and Keefer (4) in 1925 described an actively immunizing substance of a protein nature isolated from broth culture filtrates of *Pneumococcus* Type I by ultrafiltration, precipitation at a definite hydrogen ion concentration and the separation of a soluble picrate fraction. Jungeblut (5) has described an alcohol soluble substance which under certain conditions reacts specifically by precipitation with type sera. The latest studies of Julianelle (6) on skin and conjunctival hypersensitiveness in rabbits treated with products of the pneumococcus suggest the presence of a third non-type specific substance in that organism.

In this paper evidence will be presented which points to the presence of a substance other than the specific carbohydrate in the autolytic products of *Pneumococci* Type I which reacts specifically with immune serum as determined either by the precipitin reaction, or, *in vivo*, by the anaphylactic behavior of guinea pigs appropriately sensitized.

Primary Observation

The possibility of the existence of a substance quite distinct from the type specific carbohydrate was suggested by the behavior of the

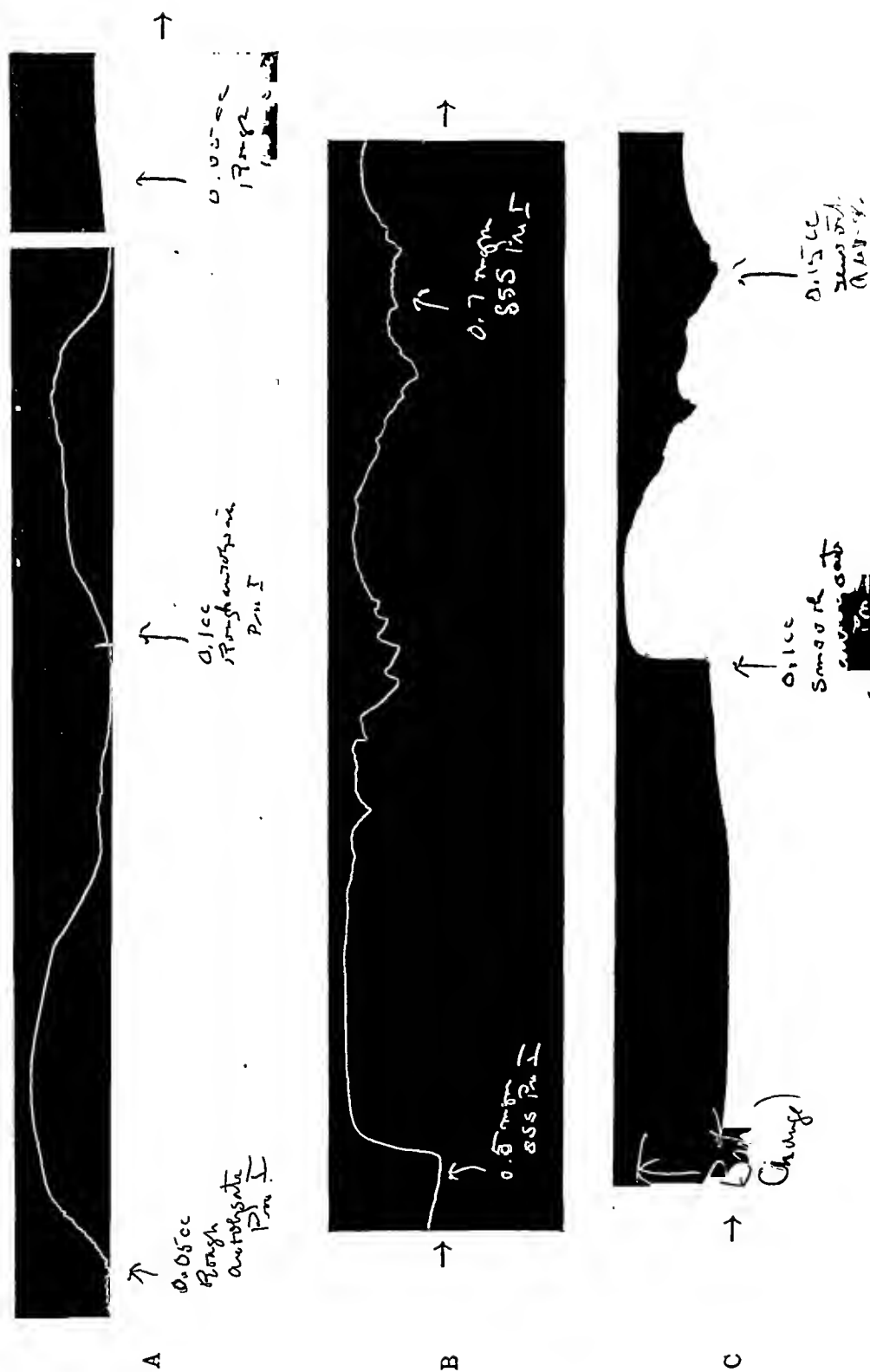


FIG. 1. The sections above represent the continuous tracing of one uterine horn of a guinea pig sensitized 24 hours before the experiment was performed by the intraperitoneal injection of 1 cc. of antipneumococcus Type I rabbit serum. In (A) is shown the effect of the autolysates from a "rough" strain of Pneumococcus Type I, in (B) that of Type I specific soluble substance, and in (C) the reaction caused by the autolysate from a "smooth" strain of Pneumococcus Type I. The volume of Ringer's solution in the bath was 25 cc.

uterine horns of guinea pigs which had been passively sensitized with antipneumococcus Type I rabbit serum. It was found that the uterine strip when treated with the following materials in the order named responded typically to each in turn:

1. Autolysate from a "rough" strain of pneumococcus.
2. The homologous specific carbohydrate purified according to the method of Heidelberger and Avery.
3. The autolysate obtained from a "smooth" strain of *Pneumococcus* Type I. (A representative tracing is reproduced in Fig. 1).

Desensitization of the uterus to each substance was demonstrated before the addition of a different material. It was also shown that in the quantities used, these materials had no comparable effect upon the uterus of a normal guinea pig. This result indicated that the autolysate obtained from the "smooth" or virulent *Pneumococci* Type I contained a substance capable of causing a typical contraction of the uterine muscle after it had been shown to be completely unresponsive to the nucleoprotein fraction (as represented by R autolysate) and to the purified specific carbohydrate. This substance has provisionally been designated the "A" substance.

The remainder of this communication consists in a description of the methods and results employed in the confirmation of the significance of this primary observation.

EXPERIMENTAL

In every instance the autolysates were produced by centrifuging, in large bottles, cultures of pneumococci grown for 24 hours in 0.1 per cent dextrose hormone broth. The sediment from about 250 cc. broth culture was taken up in 1 cc. of saline. The suspensions were collected and adjusted to contain 0.5 per cent phenol. The mixture was allowed to stand for about 72 hours at 37°C. and then kept in the ice box. The undissolved debris was centrifuged off before the clear supernatant autolysate was used in any test.

The antipneumococcus Type I rabbit serum hereafter to be designated as "normal" antipneumococcus rabbit serum was obtained in the usual manner by treating rabbits intravenously with cultures of *Pneumococci* Type I grown in broth containing 1 per cent rabbit's blood and killed by the addition of 0.3 per cent formalin.

The antipneumococcus Type I rabbit serum hereafter to be designated as anti-A *Pneumococcus* Type I rabbit serum was produced by the following procedure:

Heavy suspensions of pneumococci obtained by centrifuging 0.1 per cent dextrose hormone broth cultures, and taking up the sediment of 1 cc. saline, were treated with sufficient formalin in 10 per cent solution to give a final concentration of 0.2 per cent. After 24 to 48 hours at 37°C. tests for sterility were made. Rabbits were treated intravenously at suitable intervals with rather large amounts of the formalinized suspension.

It will be particularly noted that when heavy suspensions such as those employed are treated in the manner described, what may be spoken of as a "partial" autolysis appears to take place, *i.e.*, the organisms become Gram negative and seem to become somewhat smaller. In the case of two of the sera (228 and 230), doses of formalinized broth culture of pneumococci were given to the rabbits subsequently to the large amounts of partially autolyzed organisms. The details concerning the manner in which anti-A pneumococcus rabbit Sera 228 and 230 were prepared are given in Table I. It must be admitted that the immunological principles responsible for the production of sera which do not develop the S antibody but which do contain an antibody against the A substance are entirely unknown. Accordingly the presentation of a reliable technique for obtaining such sera cannot be given. Subsequently it will be shown that "normal" antipneumococcus Type I sera contain the A antibody which remains in the serum after the antibody reacting with the specific carbohydrate has been removed by precipitation. The most convenient and readily available method for obtaining an antiserum against the A substance depends upon this fact.

The nucleoprotein was obtained by precipitation with dilute acetic acid solution derived by dissolving pneumococci with bile according to the method of Avery and Heidelberger (7).

The specific carbohydrate was produced from pneumococci grown on 0.1 per cent dextrose hormone broth according to the methods described by Heidelberger and his associates. Owing, probably, to a slight amount of BaSO₄ in the Type I carbohydrate, the nitrogen present was equivalent to only 3.7 per cent.

Demonstration of the Presence of the A Substance in Antipneumococcus Type I Sera by Means of the Precipitin Test.—It was found that antipneumococcus rabbit sera produced by injecting the so-called "partially" autolyzed organisms gave no precipitation upon the addition of the specific carbohydrate. On the other hand, when the homologous autolysate was added, an abundant flocculent precipitate formed immediately. That this precipitate was only in a minor proportion due to the presence in the serum of a nucleoprotein antibody is shown by the results recorded in Tables II and III, which also demonstrate the absence of an antibody capable of reacting with the specific soluble substance.

A further indication that the species specific nucleoprotein is not responsible for the bulk of the precipitate obtained by adding the

TABLE I
Immunization of Rabbits Producing Anti-A Pneumococcus Serum

Serum	Days after first injection of vaccine							
	0	3	6	18	21	23	29	36
	cc.	cc.	cc.	cc.	cc.	cc.	cc.	cc.
228	0.5 F	0.5 F	0.5 F	0.5 BC	1.0 BC	2.0 BC	5.0 BC	1.0 HB
230	1.0 F	1.0 F	0.5 F	0.5 BC	1.0 BC	2.0 BC	5.0 BC	—

F = Centrifugate from 250 cc. 0.1 per cent dextrose hormone broth culture *Pneumococcus* Type I taken up in 1 cc. saline. 0.2 per cent formalin. Culture grown for 16 hours.

BC = 16 hour hormone broth culture containing 1 per cent rabbit blood. 0.2 per cent formalin.

HB = 1/10 dilution of vaccine prepared by washing growth from horse blood agar pie plate in 5 cc. 0.2 per cent formalin solution in saline.

All vaccines were injected intravenously.

TABLE II
Titration of Anti-A Pneumococcus I Rabbit Sera with the Specific Soluble Substance and the Homologous Autolysate

Antigen		1/1	1/10	1/100	1/1,000	1/10,000	1/100,000	1/1,000,000	1/2,000,000	1/4,000,000
S S S Pn. I	Serum 228	Not done	Not done	—	—	—	—	—	—	—
	Serum 230	Not done	Not done	—	—	—	—	—	—	—
Autolysate Pn. I (smooth)	Serum 228	++++	++++	++++±	++++	±	—	—	—	—
	Serum 230	++++±	++++	++++±	++++	±	—	—	—	—

++++ = very heavy precipitate falling to bottom of tube. +++ = precipitate falling to bottom of tube. ++ = heavy ring—stays up. + = definite ring. ± = ring just discernible.

The ring test technique was employed in this and the following precipitin tests in which the antigen was diluted. Readings taken after 2 hours at room temperature.

autolysate of *Pneumococcus* Type I to this serum is offered by the experiment recorded in Table IV. Type I autolysate was treated with 10 per cent acetic acid in the cold until test of a portion of the fluid indicated a pH of about 4. After standing over night in the cold, the acid-precipitable material was removed by centrifugation. The supernatant fluid was tested with acetic acid. Any further precipitate which formed after 2 hours in the ice box was removed and the supernatant fluid neutralized with sodium hydroxide. When this material was tested against an anti-"rough" *Pneumococcus* I rabbit serum

TABLE III

Titration of an Anti-A Pneumococcus Rabbit Serum, Employing Various Pneumococcus Autolysates as Antigens

Antigen	Dilution of serum						
	1/2	1/4	1/8	1/16	1/32	1/64	1/128
Pn. I autolysate (smooth).....	++++	+++±	++±	++	+	±	—
Pn. II autolysate (smooth).....	—	—	—	—	—	—	—
Pn. II autolysate (rough).....	±	—	—	—	—	—	—
Pn. III autolysate (smooth).....	±	—	—	—	—	—	—
Pn. I S S S.....	—	—	—	—	—	—	—

In this experiment the antiserum was diluted while the amount of antigen was kept constant. 1 drop of undiluted autolysate was added to 0.25 cc. of the serum dilution. 1 drop of the specific soluble substance diluted 1/10,000 was added to the same volume of diluted serum. Readings taken after 2 hours at room temperature.

which had a precipitin titre of 1:3200 (dilution of antigen) against the chemically isolated nucleoprotein, it produced no precipitate in the dilution employed. When, however, it was titrated with an anti-A serum, containing no precipitating antibody for the specific soluble substance, it showed only a slight diminution in precipitating capacity as compared with the untreated autolysate against the same serum. This diminution may in large part be accounted for by the loss of the nucleoprotein.

It may also be mentioned in this connection, although a discussion of the chemical aspects of the A substance will be temporarily deferred,

that it is possible to obtain by acid and alcohol precipitation a material which fails to give any precipitin reaction with the anti-"rough" pneumococcus serum mentioned above, but which reacts strongly with anti-A serum. This fact, taken with the evidence already presented, together with that which is to follow, makes it quite clear that the nucleoprotein fraction cannot be identified with the A substance, while the complete failure of the purified specific soluble substance to produce precipitation in these sera conclusively indicates that this substance is not the precipitating antigen concerned in the reactions.

In the light of the foregoing facts and the evidence afforded by the

TABLE IV

Titration of the A Substance before and after Precipitation with Acetic Acid of the Nucleoprotein from the Homologous Autolysate

Antigen			Dilution of antigen					
			1/8	1/32	1/128	1/512	1/2048	1/8192
Autolysate Pn. I (smooth)	{	Anti-R Pn. serum	++	+	-	-	-	-
		Anti-A Pn. I serum	++++±	+++	++±	++	+	-
Supernatant from acetic acid precipitation of autolysate Pn. I (smooth)	{	Anti-R Pn. serum	-	-	-	-	-	-
		Anti-A Pn. I serum	+++	++±	++	±	+	-

Readings taken after 2 hours at room temperature.

isolated uterus of a guinea pig sensitized with antipneumococcus Type I rabbit serum prepared in the customary manner, it became of interest to determine, if possible, by the method of specific precipitation whether or not "normal" antipneumococcus rabbit serum contains the A antibody.

A technique was devised* which enabled us to demonstrate the presence of this antibody in serum which originally contained a large amount of antibody reacting

* Dr. Hugh K. Ward in this laboratory devised this technique for eliminating a precipitating antibody from an antiserum.

with the specific carbohydrate. By titration the quantity of the purified specific soluble substance was found which caused the optimum precipitate in a "normal" antipneumococcus Type I rabbit serum. The calculated amount of carbohydrate was then added to any desired volume of the antiserum and, after 2 hours at 37°C., the precipitate was removed by centrifugation. A portion of the supernatant fluid was again titrated against varying quantities of the specific soluble substance and the optimum precipitate recorded. The calculated amount of carbohydrate causing this precipitate was added to the bulk of the supernatant fluid and after 2 hours at 37°C. the precipitate was removed. The supernatant from this precipitation was then tested with varying dilutions of the carbohydrate.

Usually no visible precipitation occurred with any dilution of the specific soluble substance employed in the tests. When tested, however, with the homologous autolysate, an abundant flocculent precipitate was observed. These results, together with those obtained with the "rough" Pneumococcus I autolysate are summarized in Table V. The autolysates of heterologous types freed from the acid precipitable nucleoprotein failed to give definite precipitates with these sera. In addition data are included which were recorded in an experiment with a therapeutic Type I antipneumococcus horse serum treated in an identical manner which was produced at the Massachusetts State Antitoxin Laboratory.

The precipitin tests with "normal" Pneumococcus Type I horse and rabbit sera lend strong support to the evidence presented by the conduct of the isolated uterus for the presence of a type specific substance in the homologous autolysate which is distinct from the specific carbohydrate.

Passive Anaphylaxis in Guinea Pigs Sensitized with Anti-A Pneumococcus Rabbit Serum.—Further proof of the existence of the A substance was sought in experiments which employed the systemic anaphylactic reaction in guinea pigs passively sensitized with anti-A rabbit serum or with a "normal" antipneumococcus rabbit serum from which the S antibody had been removed by means of the procedure described above.

It was found that guinea pigs injected intraperitoneally with 1 to 2 cc. of both anti-A rabbit sera used in the precipitin tests failed to develop symptoms of anaphylaxis upon intravenous injection of varying quantities of the purified specific carbohydrate. Nor was the antibody against the nucleoprotein or "rough" autolysate present in

sufficient concentration in these sera to confer upon guinea pigs anaphylactic sensitivity to these substances. Again, no anaphylaxis developed in animals treated with such sera when autolysates derived from either Type II or Type III virulent strains of pneumococci were introduced intravenously. The Type I anti-A sera did, however,

TABLE V

Demonstration of the A Precipitating Antibody in "Normal" Antipneumococcus Type I Sera from Which the Antibody Reacting with the Specific Substance Had Been Removed by Precipitation

Serum	Antigen	Dilution of antigen					
		1/2	1/8	1/32	1/128	1/512	1/2048
Antipneumococcus I rabbit serum	S I A	+++±	+++	++±	++	++	±±
	S II A	+	—	—	—	—	—
	S III A	±±	±	—	—	—	—
	R I A	±±	—	—	—	—	—
Antipneumococcus I horse serum	S I A (ppt.)	+++±	+++	++±	±±	+	±
	S III A (ppt.)	—	—	—	—	—	—
	R I A	++	++	+	±	—	—

S I A = Pneumococcus I autolysate (smooth).

R I A = Pneumococcus I autolysate (rough).

S II A = Pneumococcus II autolysate (smooth).

S III A = Pneumococcus III autolysate (smooth).

ppt. = Autolysate treated with dilute acetic acid in the cold to remove nucleoprotein. This was done in the case of the horse antiserum because it contained a large amount of "P" antibody.

Both horse and rabbit antisera after removal of the S S S antibody showed no precipitation with the specific soluble substance in dilutions of the latter ranging from 1/1000 to 1/4,000,000.

Ring tests—readings after 2 hours at room temperature.

regularly confer upon guinea pigs a very high degree of anaphylactic sensitivity to the autolysate derived from Type I pneumococcus. The "normal" antipneumococcus Type I rabbit serum from which the S antibody had been removed also rendered guinea pigs anaphylactically hypersensitive to the A substance in the homologous autolysate. Before the removal of the S antibody by precipitation *in vitro*, this serum

sensitized guinea pigs anaphylactically to the type specific soluble substance. After the S antibody had been eliminated, animals sensitized with this serum failed to react anaphylactically upon the injection of the carbohydrate, but did respond typically upon being injected with the autolysate from Type I virulent organisms. In addition, they showed no symptoms upon intravenous treatment with autolysate derived from a "rough" strain of Type I or from autolysates of smooth strains of Types II and III. The results of these experiments are summarized in Table VI.

Although not relevant to this subject, it is of interest to note that in these experiments with a rabbit serum which at first contained two different antibodies capable of anaphylactic sensitization, but which was deprived of one of these antibodies by precipitation with the specific antigen, the complete dependence of the anaphylactogenic antibody upon the precipitating antibody is made very clear. That these two antibodies are distinct immunological entities is still held by certain workers. There is, of course, no final proof of their identity in these experiments—a proof that can only be obtained when a chemical isolation of an antibody is accomplished. Yet the fact that the sensitizing antibody may be completely removed *in vitro* by precipitation with the specific antigen from a serum in which a second sensitizing antibody remains undisturbed is additional evidence for regarding them as one.

These anaphylaxis experiments show that in the sera of rabbits which have received injections of formalinized pneumococci an antibody develops which reacts specifically with an antigen found in the autolytic products of Type I pneumococcus. Similar materials obtained from the other two types of organism, as well as from "rough" strains of the homologous and heterologous types cause no anaphylactic symptoms. The chemically prepared nucleoprotein is likewise incapable of eliciting anaphylaxis. That the antigen in the Type I autolysate responsible for the anaphylactic symptoms is not the specific carbohydrate appears to be definitely shown not only by the failure of the latter material when pure to produce shock in guinea pigs sensitized with anti-A rabbit sera, but also by the experiments with the rabbit serum from which the S antibody originally capable of sensitizing had been removed, leaving unimpaired the serum's power to sensitize to the A substance.

TABLE VI
Anaphylaxis Experiments with Guinea Pigs Passively Sensitized with Anti-A Rabbit Sera

Guinea pig	Amount serum injected I. P.	Anti-A Pneumococcus I Rabbit Serum 228						Results
		SSS	SIA	RIA	SIIA	RIIA	SIIIA	NP
1	2 cc.	2 mg.	—	—	—	—	—	—
2	2 cc.	1 mg.	0.4 cc.	—	—	—	—	No symptoms
3	1 cc.	1 mg.	0.4 cc.	—	—	—	—	SSS = no symptoms SIA = death in 3 minutes. Autopsy typical
4	1 cc.	—	0.2 cc.	—	—	0.2 cc.	—	SSS = no symptoms SIA = death in 3 minutes. Autopsy typical
5	1 cc.	—	0.2 cc.	—	—	0.2 cc.	—	RIIA = no symptoms SIA = death in 4 minutes. Autopsy typical
6	1 cc.	—	0.2 cc.	—	0.2 cc.	—	—	RIIA = no symptoms SIA = death in 4 minutes. Autopsy typical
7	1 cc.	—	0.1 cc.	0.2 cc.	—	—	—	SIIA = no symptoms SIA = death in 3 minutes. Autopsy typical
8	1 cc.	1 mg.	0.2 cc.	—	—	—	—	RIA = no symptoms SIA = death in 3 minutes. Autopsy typical
9	1 cc.	2 mg.	0.05 cc.	—	—	0.2 cc.	—	SSS = no symptoms RIA = no symptoms SIA = death in 3 minutes. Autopsy typical
								SSS = no symptoms SIIIA = no symptoms SIA = death in 4 minutes. Autopsy typical

TABLE VI—Continued

Guinea pig	Amount serum injected I. P.	SSS	S I A	R I A	S II A	R II A	S III A	N P	Results
<i>Anti-A Pneumococcus I Rabbit Serum 230</i>									
10	1 cc.	1 mg.	0.1 cc.	0.2 cc.	—	—	—	—	SSS = no symptoms R I A = no symptoms S I A = death in 3 minutes. Autopsy typical
11	1 cc.	1 mg.	0.1 cc.	—	0.2 cc.	—	—	—	SSS = no symptoms S II A = no symptoms S I A = death in 3 minutes. Autopsy typical
12	1 cc.	1 mg.	0.1 cc.	—	—	—	0.2 cc.	—	SSS = no symptoms S III A = no definite symptoms* S I A = death in 5 minutes. Autopsy typical
13	1 cc.	—	0.2 cc.	—	—	—	—	3 mg.	N P = no symptoms S I A = death in 3 minutes. Autopsy typical
<i>Anti-Pneumococcus I Rabbit Serum without SSS Antibody</i>									
14	1 cc.	2 mg.	0.2 cc.	0.2 cc.	—	—	—	—	SSS = no symptoms R I A = no symptoms S I A = death in 3 minutes. Autopsy typical
15	1 cc.	1 mg.	0.1 cc.	—	—	—	—	—	SSS = no symptoms S III A = no symptoms S I A = death in 3 minutes. Autopsy typical
16	1 cc.	1 mg.	0.1 cc.	—	0.2 cc.	—	—	—	SSS = no symptoms S II A = no symptoms S I A = death in 6 minutes. Autopsy typical

S S S = Pneumococcus I specific soluble substance.
 S I A = Pneumococcus I (smooth) autolysate.
 R I A = Pneumococcus I (rough) autolysate.
 S II A = Pneumococcus II (smooth) autolysate.
 R II A = Pneumococcus II (rough) autolysate.
 S III A = Pneumococcus III (smooth) autolysate.
 N P = Pneumococcus I nucleoprotein.
 The sensitizing serum was injected intraperitoneally 24 hours before the test substances were administered intravenously

materials. In every case the specific soluble substance was allowed to elapse between the intravenous injection of the various test or nucleoprotein. The Pneumococcus I (smooth) autolysate was injected first. This was followed by the non-specific autolysate the amounts used in these experiments produced no symptoms in normal guinea pigs. The Pneumococcus I (smooth) autolysate in * Guinea pig 12 after the injection of Type III autolysate showed weakness and rapid respiration. Recovery was immediate. These symptoms were not interpreted as being anaphylactic.

The autolysate prepared from a different strain of virulent Type I pneumococcus was tested both by the methods of precipitation and of anaphylaxis for the A substance, which was found to be present.

TABLE VII
Effect of Heat on the A Substance

		Dilution of antigen					
		1/8	1/32	1/128	1/512	1/2048	1/8192
Anti-A Pneumococcus Type I rabbit serum 228	Untreated phenolized S I A	+++±	+++	+++	+++	+++	+++
	Supernatant after treatment with HAc	+++	+++	++	±	+	-
	HAc supernatant boiled at pH 9	±	-	-	-	-	-
	HAc supernatant boiled at pH 4	+++	+++	++	±	+	-
	HAc supernatant autoclaved at pH 9	-	-	-	-	-	-
	HAc supernatant autoclaved at pH 4	+*	±*	-	-	-	-
Anti-R Pneumococcus Type I rabbit serum	Untreated phenolized S I A	++	+	-	-	-	-
	Supernatant after treatment with HAc	-	-	-	-	-	-
	HAc supernatant boiled at pH 9	-	-	-	-	-	-
	HAc supernatant boiled at pH 4	-	-	-	-	-	-
	HAc supernatant autoclaved at pH 9	-	-	-	-	-	-
	HAc supernatant autoclaved at pH 4	±*	-	-	-	-	-

* These reactions may probably be interpreted as non-specific, since the HAc supernatant did not give this reaction with anti-R serum before autoclaving at pH 4.

S I A = Pneumococcus Type I (smooth) autolysate.

HAc = Acetic acid.

Solutions boiled for $\frac{1}{2}$ hour.

Solutions autoclaved for 1 hour at 15 pounds pressure.

The ring test was employed in this experiment. Readings after 2 hours at room temperature.

Physical and Chemical Characteristics of the A Substance

Effect of Heat.—A characteristic of the A substance which sharply distinguishes it from the soluble specific substance is the destructive

effect of heat upon it when in weakly alkaline solution. If boiled for $\frac{1}{2}$ hour at pH 9, its effectiveness as a precipitating antigen is reduced at least one thousand fold, while the specific carbohydrate remains unchanged. On the other hand, when the reaction is adjusted to pH 4 with 10 per cent acetic acid, a solution containing the A substance may be boiled for the same period of time without losing its activity. In both acid and alkaline solution, autoclaving for 1 hour at 15 pounds pressure practically destroys its effectiveness. In the experimental results recorded in Table VII, the evidence for these statements is presented. To eliminate in so far as is possible the nucleoprotein fraction, the autolysate used in these experiments was

TABLE VIII
Effect of Peptic Digestion on A Substance

Serum	Dilution of the antigen				
	1/1	1/8	1/32	1/128	1/152
Anti-A Pneumococcus I rabbit serum 228	After digestion	+++	++	±	+
	Control (undigested)	+++	++	±	+
Anti-R Pneumococcus rab- bit serum	After digestion	—	—	—	—
	Control (undigested)	++	±	—	—

Readings taken after 2 hours at room temperature.

first treated with acetic acid and the resulting precipitate discarded before the supernatant fluid containing the A substance was either boiled or autoclaved.

Effect of Peptic and Tryptic Digestion.—Digestion for 72 hours in solution containing 1 per cent pepsin at 37.5°C. does not decrease the precipitating power of the A substance. To 1 cc. of Pneumococcus I autolysate, 1 cc. of 2 per cent solution of pepsin in 0.1 N hydrochloric acid was added. The mixture was then placed in a "celofane" dialyzing bag and immersed in a solution of 0.1 N hydrochloric acid. The liquid outside the bag was changed twice each day. As a control a similar mixture of autolysate and boiled pepsin solution was dialyzed under the same conditions. The enzyme in a concentration of 1 per

cent readily digested coagulated egg white (Mett tube). Its activity was also indicated by the reduction in the precipitating strength of the digest when added to an anti-R pneumococcus rabbit serum. This loss of precipitating capacity is, no doubt, to be attributed to the digestion of the nucleoprotein by the enzyme. In Table VIII are presented the results of an experiment to determine the effect of pepsin on the A substance.

Because of the fact that in weakly alkaline solution the activity of the A substance seems to be markedly impaired by prolonged exposure to a temperature of 37.5°C., it was not possible to determine so clearly the effect of tryptic digestion on it. It may be stated, however, that a solution of the A substance exposed to the action of this enzyme loses no more of its capacity to react with an A antiserum than does a control solution to which no trypsin has been added but which has been subjected to identical conditions.

Chemistry of the A Substance.—Until the completion of a study now being pursued which is concerned with the isolation and properties of this substance, little can be definitely stated in respect to its chemistry. In many of its reactions it resembles the specific soluble substance. Precipitated by the same volume of alcohol, traces of it are to be found in that material until very near the end of the purifying process. It is precipitated by phosphotungstic acid and by silver nitrate. It is not precipitated either by acetic or hydrochloric acid. Until isolated in a state at least approaching purity, the tests for protein have, of course, no meaning.

DISCUSSION

In the preceding sections experimental evidence has been presented which leads to the conclusion that in the autolytic products of Pneumococcus Type-I there is to be found a substance of unknown chemical composition reacting specifically both in precipitin and anaphylactic reactions with sera produced by injecting rabbits with formalinized pneumococci. This substance is distinguished from the specific carbohydrate not only by its specific precipitating and anaphylactic action with such sera, but also by its instability when boiled in weakly alkaline solution. Although this last characteristic is analogous to the conduct of certain proteins which undergo racemization under similar

conditions, the fact that pepsin and, less clearly, trypsin do not digest the A substance renders exceedingly doubtful its ultimate identification as a protein. At present the chemical aspects of the problem are being investigated.

The hypothesis of Landsteiner concerning the "haptene" or partial antigen so conclusively confirmed experimentally in the case of the lipoidal antigen of Forssmann and the bacterial carbohydrates, must inevitably be considered in relation to any substance which reacts *in vitro* with an antiserum. Since the chemical isolation of the A substance has not yet been accomplished, it is obviously impossible to arrive at any definite conception in respect to its function as a complete or partial antigen. *A priori*, however, the facts mentioned above, which suggest a substance of non-protein nature, also make it unlikely that the A antigen *per se* possesses the capacity to stimulate the production of antibody when injected into an animal.

The work of Reimann (9) and others has shown that immunization with the autolytic products of the pneumococcus produces an antibody reacting only with the nucleoprotein fraction. Although their results do not definitely preclude the possibility that an A antibody was also present in small concentration, they render it improbable. Had an A antibody been present in a serum along with the nucleoprotein antibody, that serum would have exhibited a higher precipitin titre against the homologous autolysate than against heterologous autolysates. Actually, it was found that in the sera of an animal immunized with the autolysate from any pneumococcus or with the nucleoprotein fraction the precipitin titre was the same whether the homologous or heterologous autolysate was employed as antigen.

We have not attempted an extensive series of experiments to determine whether or not an antibody against the A substance developed in the sera of rabbits immunized with autolysate derived from *Pneumococcus* Type I. In the sera of three animals treated in this way, however, no antibody against the A substance was demonstrated. Thus, until further data are available, the preponderance of evidence is in favor of regarding the A substance as belonging to the order of haptenes.

Whether or not materials analogous to the A substance in *Pneumo-*

coccus Type I exist in the autolytic products of Pneumococci Type II and Type III has not yet been definitely shown.

CONCLUSIONS

1. Evidence has been presented for the existence of a substance distinct from the specific carbohydrate in the autolytic products of Pneumococcus Type I.

2. The substance reacts specifically by precipitating homologous antiserum which either occurs naturally without antibody against the specific carbohydrate or has been deprived of that antibody artificially.

3. In guinea pigs passively sensitized with such antisera the homologous autolysate containing the substance alone produces typical lethal anaphylactic shock.

4. In weakly alkaline solution the substance is destroyed by boiling. In weakly acid solution it resists a temperature of 100°C. for at least $\frac{1}{2}$ hour. Autoclaving for 1 hour at 15 pounds pressure in either acid or alkaline solution destroys its activity as precipitinogen.

5. The substance is resistant to peptic digestion.

6. The chemical nature and the possible identification of the substance as a haptene have been discussed.

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THE INFLUENCE OF ULTRA-VIOLET RADIATION ON THE WEIGHT OF ADULT RABBITS, NORMAL AND SYPHILITIC

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During the course of an experiment previously reported (1), it was noted that rabbits exposed to ultra-violet radiation under certain conditions did not appear to be in as good physical condition as animals living in an environment from which all light was excluded and were more susceptible to intercurrent infections. With these facts in mind, the experiment was repeated with a view to determining the influence of ultra-violet irradiation on body weight, the chemical composition of the blood, and susceptibility to intercurrent infections of normal and syphilitic animals and on the reaction to syphilitic infection. The present paper will be limited to a report of the results obtained for body weight and susceptibility to intercurrent infections.

Material and Methods

In the experiment to be reported, 60 male rabbits between 6 and 8 months of age were used. The animals were divided into 6 comparable groups of 10 each, and from the beginning of the experiment on November 27, 1928, until June 15, 1929, these animals were kept in a room from which all light was excluded. They were caged separately, and fed the routine diet of hay, oats, and cabbage. The temperature of the dark room was satisfactorily maintained at 70° to 75°F. and the humidity varied with that of the outside air. The animals were weighed at weekly intervals.

Beginning December 5, 1928, 30 animals, listed as Groups II, IV, and VI were exposed for an hour each day to the unfiltered radiations of a quartz-mercury arc lamp (80 volts, 4.2 amperes) at a distance of 4.5 meters. The doors of the cages were open to permit free access of light but no depilatory measures were used and at no time throughout the experiment could there be detected evidence of derma-

titis, conjunctivitis, or any other inflammatory process which might be attributed to the action of ultra-violet radiation. The other animals, Groups I, III, and V, remained in the dark room throughout the experiment. Group I served as a control for Groups III and V, and Group II, as the control for Groups IV and VI.

A summary of experimental procedures, according to groups, is given in the following table:

Group	Number of animals	Dark room	Ultra-violet irradiation started	Inoculated with <i>T. pallidum</i>	Termination of experiment
I	10	Nov. 27, 1928			June 15, 1929
II	10	Nov. 27, 1928	Dec. 5, 1928		June 15, 1929
III	10	Nov. 27, 1928		Dec. 18, 1928	June 15, 1929
IV	10	Nov. 27, 1928	Dec. 5, 1928	Dec. 18, 1928	June 15, 1929
V	10	Nov. 27, 1928		Jan. 24, 1929	June 15, 1929
VI	10	Nov. 27, 1928	Dec. 5, 1928	Jan. 24, 1929	June 15, 1929

All animals received some light from two other sources. For a brief period each day a 30 Watt amber tinted Mazda lamp was used in the dark room for cleaning cages, feeding, and making certain necessary observations. The animals were also exposed to diffuse filtered sunlight for a brief period once every 2 weeks when they were brought into the laboratory for bleeding purposes. During the remaining time all animals were kept in the dark room.

The mean values contained in Tables I to VI inclusive have been smoothed by the formula $\frac{a + 2b + c}{4}$ and are presented in the graphs (Text-figs. 1 to 3) in terms of per cent variation from a standard mean value, using for this purpose 2200 gm.

RESULTS

The results of the observations made in this experiment are presented in the form of tabulated summaries, Tables I to VIII, which are supplemented by a series of graphs, Text-figs. 1 to 3.

TABLE I
Gross Body Weight. Normal Dark Room

Date	Group mean	Smoothed net variation	Per cent variation
<i>1928-29</i>	<i>gm.</i>	<i>gm.</i>	
Nov. 30	1940	-260	-11.8
Dec. 6	2043	-167	- 7.6
Dec. 12	2088	-113	- 5.1
Dec. 20	2132	- 82	- 3.7
Dec. 28	2121	- 61	- 2.8
Jan. 5	2182	- 21	- 0.9
Jan. 12	2232	+ 17	+ 0.8
Jan. 18	2222	+ 32	+ 1.5
Jan. 25	2253	+ 59	+ 2.7
Feb. 5	2309	+105	+ 4.8
Feb. 9	2350	+154	+ 7.0
Feb. 16	2409	+199	+ 9.0
Feb. 22	2431	+186	+ 8.8
Mar. 6	2273	+117	+ 5.3
Mar. 16	2290	+ 94	+ 4.3
Mar. 23	2325	+114	+ 5.2
Mar. 29	2315	+ 88	+ 4.4
Apr. 6	2197	+ 33	+ 1.5
Apr. 14	2225	+ 25	+ 1.1
Apr. 19	2262	+ 61	+ 2.8
Apr. 27	2295	+ 98	+ 4.5
May 11	2342	+138	+ 6.3
May 17	2355	+103	+ 4.7
May 25	2162	- 1	0
June 1	2120	- 58	- 2.6
June 8	2167	- 37	- 1.7
June 15	2200	0	0
Group mean.....	2231	+ 31	+ 1.4

Dec. 6 to Jan. 25—14.5.

Mean gain—13.2.

Final net gain—11.8.

TABLE II
Gross Body Weight. Normal Ultra-Violet

Date	Group mean	Smoothed net variation	Per cent variation
<i>1928-29</i>	<i>gm.</i>	<i>gm.</i>	
Nov. 30	1865	-335	-15.7
Dec. 6	1970	-194	- 8.8
Dec. 12	1998	-150	- 7.0
Dec. 20	2084	-133	- 6.0
Dec. 28	2103	- 77	- 3.5
Jan. 5	2216	- 10	- 0.4
Jan. 12	2228	+ 34	+ 1.5
Jan. 18	2265	+ 46	+ 2.1
Jan. 25	2236	+ 59	+ 2.6
Feb. 5	2322	+ 75	+ 3.4
Feb. 9
Feb. 16	2220	+104	+ 4.7
Feb. 22
Mar. 6	2453	+186	+ 8.8
Mar. 16	2420	+106	+ 4.8
Mar. 23
Mar. 29
Apr. 6
Apr. 14	2031	- 63	- 2.9
Apr. 19
Apr. 27	2065	-133	- 6.0
May 11	2109	- 81	- 3.6
May 17	2193	- 31	- 1.3
May 25	2180	- 47	- 2.1
June 1	2059	- 98	- 4.5
June 8	2112	-114	- 5.2
June 15	2063	-137	- 6.2
Group mean.....	2152	- 48	- 2.2

Dec. 6 to Jan. 25—18.3.

Mean gain—13.5.

Final net gain—9.5.

TABLE III

Gross Body Weight. Dark Room. T. pallidum Inoculated on December 12, 1928

Date	Group mean	Smoothed net variation	Per cent variation
1928-29	gm.	gm.	
Nov. 30	1902	-197	- 8.9
Dec. 6	1955	-119	- 5.4
Dec. 12	2180	- 98	- 4.5
Dec. 20	2000	- 63	- 2.9
Dec. 28	2230	+ 31	+ 1.3
Jan. 5	2240	+ 27	+ 1.2
Jan. 12	2342	+168	+ 7.6
Jan. 18	2387	+174	+ 7.9
Jan. 25	2357	+193	+ 8.8
Feb. 5	2397	+211	+ 9.6
Feb. 9	2422	+211	+ 9.6
Feb. 16	2405	+230	+10.5
Feb. 22	2415	+247	+11.2
Mar. 6	2485	+230	+10.5
Mar. 16	2405	+213	+ 9.7
Mar. 23	2425	+214	+ 9.7
Mar. 29	2417	+213	+ 9.7
Apr. 6	2400	+245	+11.2
Apr. 14	2425	+274	+12.4
Apr. 19	2470	+298	+13.5
Apr. 27	2500	+327	+14.9
May 11	2500	+321	+14.8
May 17	2567	+240	+10.9
May 25	2450	+108	+ 4.8
June 1	2295	+ 25	+ 1.1
June 8	2215	+ 6	+ 0.2
June 15	2200	0	0
Group mean.....	2333	+133	+ 6.0

TABLE IV

Gross Body Weight. Ultra-Violet. T. pallidum Inoculated on December 12, 1928

Date	Group mean	Smoothed net variation	Per cent variation
<i>1928-29</i>	<i>gm.</i>	<i>gm.</i>	
Nov. 30	1852	-248	-11.2
Dec. 6	1900	-183	- 8.7
Dec. 12	2017	-101	- 4.6
Dec. 20	2065	-132	- 6.0
Dec. 28	2127	- 87	- 4.3
Jan. 5	2135	- 56	- 2.4
Jan. 12	2180	- 21	- 0.9
Jan. 18	2212	+ 12	+ 0.5
Jan. 25	2245	+ 40	+ 1.8
Feb. 5	2260	+ 41	+ 1.8
Feb. 9	2212	+ 14	+ 0.6
Feb. 16
Feb. 22	2175	- 24	- 1.1
Mar. 6
Mar. 16	2134	- 66	- 3.0
Mar. 23	2093	- 87	- 3.9
Mar. 29	2142	- 67	- 3.0
Apr. 6	2156	- 53	- 2.4
Apr. 14	2134	- 71	- 3.2
Apr. 19
Apr. 27
May 11	2090	-108	- 4.9
May 17
May 25
June 1	2053	-147	- 6.7
June 8
June 15
Group mean.....	2115	- 85	- 3.9

TABLE V

Gross Body Weight. Dark Room. T. pallidum Inoculated on January 24, 1929

Date	Group mean	Smoothed net variation	Per cent variation
1928-29	gm.	gm.	
Nov. 30	1932	-268	-12.2
Dec. 6	2162	-178	- 8.1
Dec. 12	2222	+ 15	+ 0.7
Dec. 20	2255	+ 56	+ 2.4
Dec. 28	2295	+ 91	+ 4.1
Jan. 5	2325	+130	+ 5.9
Jan. 12	2380	+174	+ 7.9
Jan. 18	2412	+212	+ 9.6
Jan. 25	2447	+245	+11.1
Feb. 5	2477	+274	+12.4
Feb. 9	2497	+303	+13.8
Feb. 16	2542	+305	+13.9
Feb. 22	2442	+254	+11.5
Mar. 6	2387	+208	+ 9.5
Mar. 16	2417	+204	+ 9.4
Mar. 23	2395	+191	+ 8.7
Mar. 29	2357	+130	+ 5.9
Apr. 6	2312	+128	+ 5.8
Apr. 14	2332	+127	+ 5.8
Apr. 19	2332	+146	+ 6.6
Apr. 27	2391	+163	+ 7.5
May 11	2339	+148	+ 6.7
May 17	2322	+103	+ 4.7
May 25	2230	+ 50	+ 2.3
June 1	2218	+ 44	+ 2.0
June 8	2290	+ 90	+ 4.1
June 15
Group mean.....	2335	+135	+ 6.1

TABLE VI

Gross Body Weight. Ultra-Violet. T. pallidum Inoculated on January 24, 1929

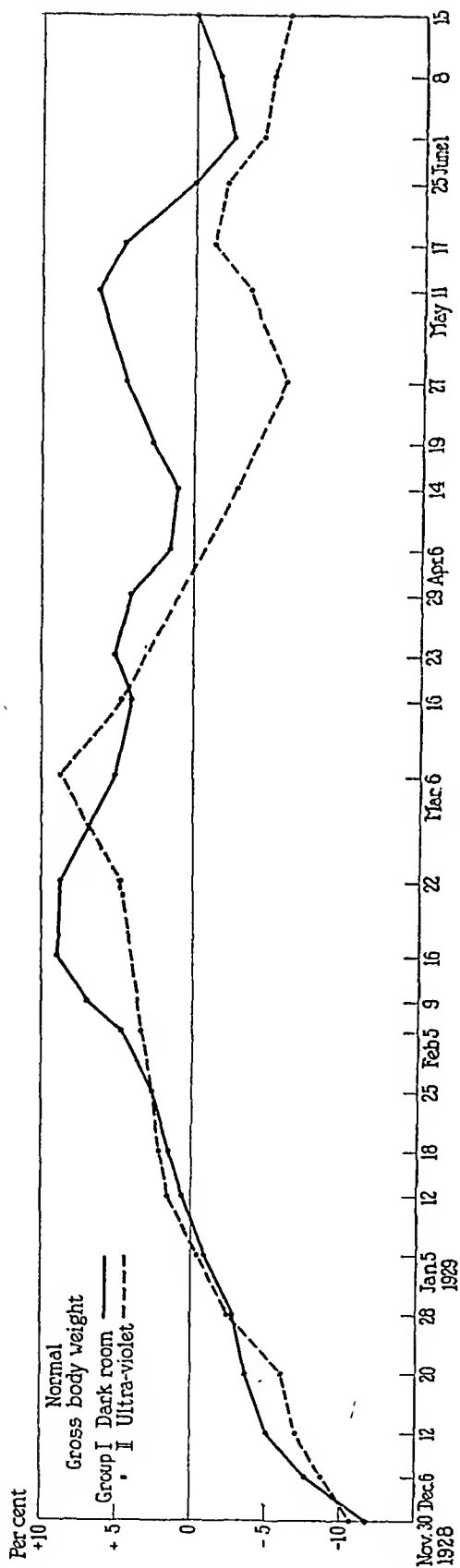
Date	Group mean	Smoothed net variation	Per cent variation
<i>1928-29</i>	<i>gm.</i>	<i>gm.</i>	
Nov. 30	1922	-258	-11.5
Dec. 6	2061	-168	- 7.6
Dec. 12	2086	- 96	- 4.2
Dec. 20	2183	- 39	- 1.7
Dec. 28
Jan. 5	2194	- 2	- 0.1
Jan. 12	2222	+ 22	+ 1.0
Jan. 18	2250	+ 48	+ 2.2
Jan. 25	2269	+ 75	+ 3.4
Feb. 5	2303	+ 81	+ 3.7
Feb. 9	2250	+ 68	+ 3.1
Feb. 16	2272	+ 69	+ 3.1
Feb. 22	2266	+ 65	+ 3.0
Mar. 6	2267	+ 69	+ 3.1
Mar. 16	2277	+ 74	+ 3.4
Mar. 23
Mar. 29	2275	+ 45	+ 2.0
Apr. 6
Apr. 14
Apr. 19	2155	+ 40	+ 1.9
Apr. 27
May 11
May 17	2025	- 48	- 2.2
May 25
June 1	2002	-148	- 6.7
June 8	1922	-212	- 9.6
June 15
Group mean.....	2168	- 32	- 1.5

TABLE VII
Per Cent Gain from Initial Weight

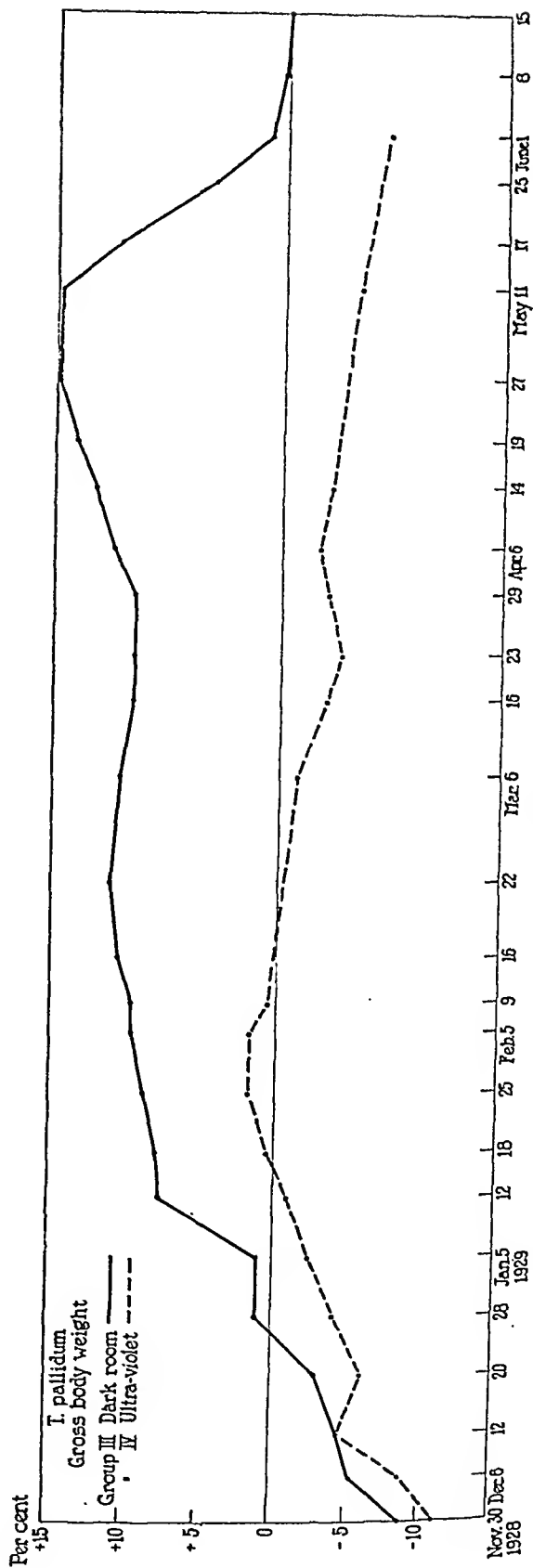
Dark room				Ultra-violet			
Group	Jan. 25, 1929	Mean	Final	Group	Jan. 25, 1929	Mean	Final
I	16.1	15.0	13.4	II	19.9	15.4	10.6
III	24.8	22.6	15.1	IV	21.2	14.2	10.8
V	26.6	20.8	18.5	VI	18.1	12.7	0.0

TABLE VIII

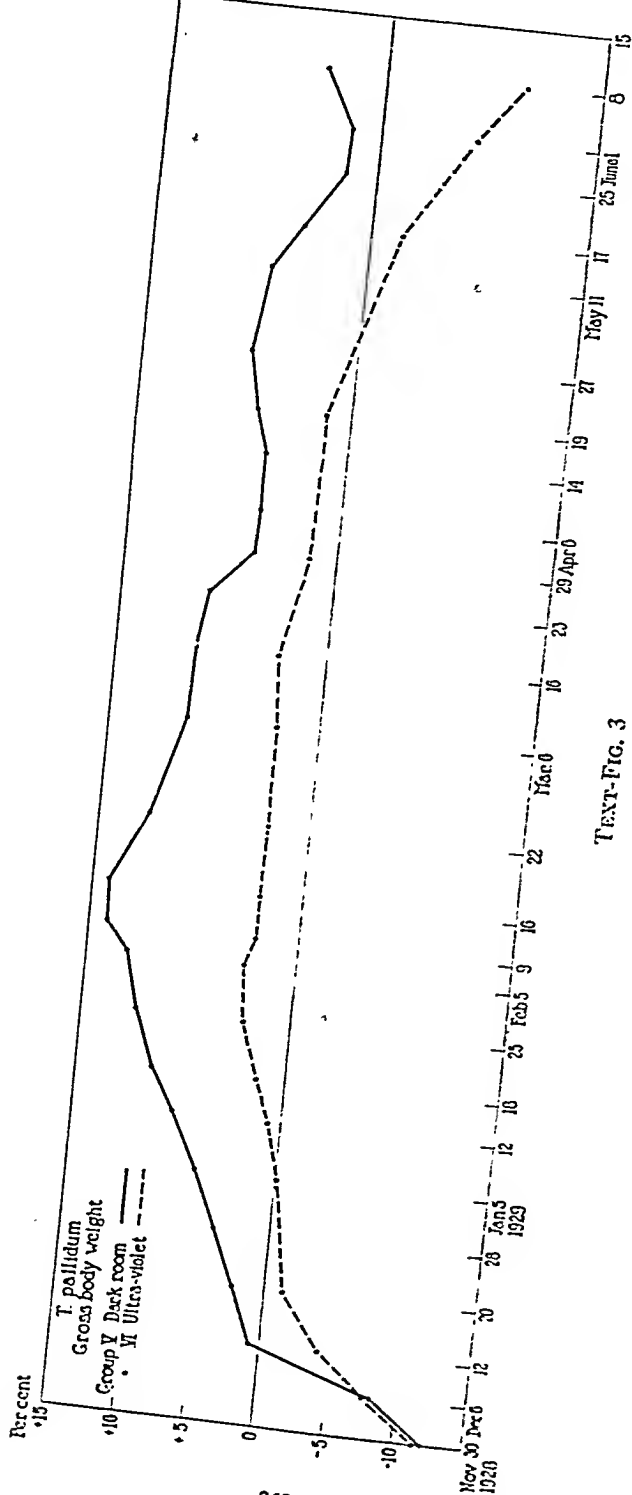
Dark room		Ultra-violet	
Group	Survivals	Group	Survivals
I	10	II	6
III	10	IV	8
V	9	VI	9



TEXT-FIG. 1



TEXT-FIG. 2



TEXT-FIG. 3

DISCUSSION AND CONCLUSIONS

In taking up the discussion of the results recorded above, attention should be directed to the fact that in these experiments the only light of any consequence which was received by the irradiated animals was that derived from the quartz mercury lamp. These experiments differ, therefore, from others in which animals receiving unfiltered or diffuse filtered sunlight are given an additional ultra-violet irradiation. A further condition to which attention should be called is the fact that during the course of these experiments, there was a marked deterioration of the lamp used, so that the energy delivered during the latter part of the experiment was much less than at the beginning. It is regretted that the actual change and the rate of decrease can not be given accurately. But throughout the entire period of the experiment, all animals lived in a room from which all extraneous light was excluded, the only environmental variation being the exposure of Groups II, IV, and VI to ultra-violet radiation for an hour each day at a distance of 4.5 meters.

Considering the results for normal animals first, it will be seen by a comparison of the figures for body weight that from the beginning of the experiment on November 30, 1928, until January 25, 1929, both Groups I and II showed a gradual increase in body weight. Following this, the dark group exhibited a rather sharp increase in weight reaching a maximum on February 16. The increase in the ultra-violet group of animals was more gradual, their maximum weight being attained on March 6. From these high values both groups showed a loss in weight, the group exposed to ultra-violet radiation losing more than animals living in total darkness. After March 16 and throughout the remainder of the experiment, the general level of body weight of the ultra-violet group was below that of the dark group. The latter group exhibited a secondary increase in weight during the first week of May, this same increase being reflected in the ultra-violet animals although at a much lower level. From these levels, both groups decreased in weight, the ultra-violet animals showing a more gradual decline while the dark group showed a slight increase in weight during the last 2 weeks of the experiment.

The difference between the two groups of animals with respect to

susceptibility to intercurrent disease (Table VIII) is in agreement with the results obtained for body weight. This is clearly shown by the occurrence of 4 deaths among the irradiated animals and none among those which received no ultra-violet light. Postmortem examination showed that death was due in all cases to pneumonia.

The effects of irradiation on syphilitic rabbits were essentially the same as on normal animals. The animals of Groups III and IV (Tables III and IV; Text-fig. 2) were inoculated with the Nichols strain of *T. pallidum* on December 24, 1928. All other conditions were the same as for Groups I and II. Except for the values obtained on December 12, at which time both groups coincided, the gross body weights for animals exposed to ultra-violet radiation was far below that maintained by animals living in total darkness. On May 6, there was a difference of 20 per cent between the mean values obtained for these two groups of animals. While the course of syphilis in the two groups of animals was moderately severe, yet no deaths were recorded as resulting from the specific infection. However, at the end of the experiment, all animals of Group III were alive while 2 died in Group IV, deaths being due to pneumonia.

The animals in Groups V and VI were inoculated on January 24, 1929, with the same strain of *T. pallidum* as were Groups III and IV. In general, it can be stated that the syphilitic infection in these animals was less severe than that found in Groups III and IV. The mortality rate from non-specific infection was the same in both Groups V and VI.

From the beginning of the experiment until December 6, the animals in Group VI showed a higher gross body weight than those in Group V, but from this time on the animals in Group VI maintained a level below that of Group V. In these two groups of animals, it will be noted that the difference in weight is not as great as that found to exist between Groups III and IV.

If the gain in weight is calculated in per cent of the original or first recorded weights (Table VII), essentially the same results are demonstrable. At the end of the first 7 weeks of the experiment, January 25, 1929, the animals in Group II showed a greater per cent gain in weight than did the control animals in Group I. It will also be noted that the mean per cent gain was slightly higher for animals exposed to ultra-violet radiation than for those animals in the dark room. The final

weight for the ultra-violet group was less than for those animals in the dark room. In the 4 other groups the per cent gain was less in animals exposed to ultra-violet light than in those remaining in total darkness; in Group VI the final weight was exactly the same as the original.

Another factor which influences all calculations is the mortality rate among these 6 groups of animals (Table VIII). Seven deaths occurred among animals remaining in the dark room. The deaths in each group were due to pneumonic infections.

SUMMARY AND CONCLUSION

In summarizing the results obtained for these 6 groups of animals, the following conclusions may be drawn:

Normal rabbits living in total darkness and exposed to ultra-violet radiation at regular intervals showed a more rapid rate of increase in weight than animals living under the same condition, but after an initial period of rapid increase, the irradiated animals maintained a lower body weight than those living in the dark.

Under the same conditions, animals inoculated with *T. pallidum* and exposed to ultra-violet light maintained a lower weight than the corresponding control groups living in total darkness.

Furthermore, the mortality rate from pneumonic infection was found to be greater in animals exposed to ultra-violet radiation than in those living entirely in the dark.

It is evident, therefore, that, under the conditions given, ultra-violet radiation was detrimental rather than beneficial.

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A STUDY OF THE RESISTANCE OF NORMAL HUMAN BEINGS TO RECENTLY ISOLATED STRAINS OF PATHOGENIC PNEUMOCOCCI*

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That man must be fairly resistant to the pneumococcus is attested by the relative infrequency of known contact infection among those attending pneumonia patients, despite the fact that under such conditions pneumococci are probably often inhaled in considerable numbers. When contact infection has been observed it is almost always with *Pneumococcus* Types I and II from which it might be inferred that these two types are the most virulent for man. But we have no definite information as to the relative human virulence of different strains or types of pathogenic pneumococci and but little data concerning variations in individual susceptibility to a single strain.

While a direct approach to this problem is not possible for obvious reasons, an indirect method of study has become available as a result of experimental observation in animals. It was found that the resistance of an animal to pneumococcus infection was paralleled very closely by the pneumococidal power of the blood. When the serum and leucocytes of a single species, the rabbit, were tested against pneumococci of varying rabbit virulence, the pneumococci of low virulence were destroyed in considerable numbers while those of high virulence grew readily in the serum-leucocyte mixtures. Again the serum and leucocytes of young rabbits which are highly susceptible to pneumococci of low virulence for the adult rabbit, were found to possess no pneumococidal action whatsoever. There was found to be a general quantitative relationship between the killing dose of pneumococci and the pneumococidal power of the animal's blood (1, 2).

* Reported in brief in the *Proceedings of the Institute of Medicine of Chicago*, 1929, 7, 224.

Method and Materials

The pneumococcal tests were carried out as already described (1, 3) employing quantitated mixtures of serum, leucocytes, and pneumococci in sealed tubes which were agitated during incubation. Pooled normal serum in each test consisted of a mixture of serum secured from four to seven persons.¹ Human leucocytes were obtained from Group I (Jansky) donors. Serum and leucocytes from the same person were often used in testing single individuals. The strains of pneumococci employed were those freshly isolated from cases of lobar pneumonia. Frequently they were used after only one transfer in broth. The standard suspension was one billion organisms per cubic centimeter. Tests for opsonins and agglutinins were made as detailed in a previous study (4).

We are much indebted to Miss Georgia Cooper for diagnostic sera which enabled us to classify strains of atypical Type II and Group IV pneumococci.

EXPERIMENTAL

Pneumococcal Action of Normal Human Serum-Leucocyte Mixtures

The tests were carried out as shown in Table I. From one to four pneumococcus strains were tested at a time and the tests were repeated in order to secure comparative results. It is seen in this experiment that the pneumococci of all four strains were killed in relatively large numbers by the normal human serum and leucocytes. Observations on 26 strains studied in this way are summarized in Table II. While considerable variation in susceptibility to the killing action of the serum-leucocyte mixtures was found to exist among the different members of the series, none were able to survive or grow in quantities less than 10^{-5} of the standard suspension. The variations in growth capacity appeared to be independent of type nor did it seem to signify whether the pneumococci were isolated from the blood, lung or sputum, in recovering or fatal cases, early or late in the disease. An interpretation of these results on the basis of the much more complete observations in animals cited above, indicates that normal human beings as a group possess a considerable degree of natural immunity against pathogenic pneumococci.

¹ It was not possible to get an accurate past history from all of them as to antecedent occurrence of pneumonia but judging by a number of tests on persons of known negative past history, the inclusion of individuals who may have had pneumonia years previously could have made no material difference in the results.

TABLE I

Pneumococcal Action of Normal Human Serum and Leucocytes for Recently Isolated Pneumococci

Human serum* 0.2 cc. + leucocyte suspension 0.1 cc. + pneumococcus suspension 0.1 cc. + gelatin Locke's solution 0.1 cc.

Type	Approximate number of pneumococci	Growth as shown by methemoglobin formation at			Survival of pneumococci at 72 hours
		15 hrs.	42 hrs.	72 hrs.	
(Sputum)	1,000,000(10^{-3})	+	+++	++++	+
	100,000(10^{-4})	0	0	0	0
	10,000(10^{-5})	0	0	0	0
	1,000(10^{-6})	0	0	0	0
	100(10^{-7})	0	0	0	0
II (Blood)	1,000,000(10^{-3})	0	+	+++	+
	100,000(10^{-4})	0	+	+++	+
	10,000(10^{-5})	0	0	0	0
	1,000(10^{-6})	0	0	0	0
IIa (Blood)	1,000,000(10^{-3})	0	++	++++	+
	100,000(10^{-4})	0	0	0	0
	10,000(10^{-5})	0	0	0	0
	1,000(10^{-6})	0	0	0	0
III (Lung)	1,000,000(10^{-3})	0	+	+++	+
	100,000(10^{-4})	0	0	+++	+
	10,000(10^{-5})	0	0	0	0
	1,000(10^{-6})	0	0	0	0

Controls with serum only**

I	100(10^{-7})	++++			+
II	100(10^{-7})	++++			+
IIa	100(10^{-7})	+++			+
III	100(10^{-7})	+			+

Plates of 10^{-7} dilution of the pneumococcus suspensions

I	No. of colonies	114
II	" " "	50
IIa	" " "	41
III	" " "	90

* Pooled serum.

** 0.1 cc. Laked red blood cells added.

The virulence of the pneumococci for mice was found to parallel in a general way their capacity to grow in the human serum-leucocyte

TABLE II

Pneumococcal and Opsonic Action of Normal Human Serum and Leucocytes for Recently Isolated Pneumococci

Case	Type	Pneumococci isolated		Outcome of disease	Action of normal serum and leucocytes		
		From	Day of disease		Pneumococ- cidal Largest amt. susp. killed	Opsonic	Agglutina- tion
R. S.....	I	Sputum	10th	Rec'd	10^{-4}	—	+
L. R. E.....	I	Blood	4th	Died	10^{-4}	—	—
J. L.....	I	Sputum	4th	Rec'd	10^{-4}	+	+++
A. L.....	I	Sputum	6th	Rec'd	10^{-3}	—	—
W. Mc.....	I	Lung	5th	Rec'd	10^{-3}	—	—
J. T. A.....	I	Sputum	1st	Rec'd	10^{-5}	—	—
J. V.....	II	Blood	2nd	Died	10^{-5}	+++++	+++++
S. P.....	II	Blood	7th	Died	10^{-5}	—	+++++
C. McN.....	II	Sputum	3rd	Died	10^{-5}	+	++
M. M.....	II	Sputum	7th	Rec'd	10^{-4}	+	++
N. S.....	II	Sputum	4th	Rec'd	10^{-3}	+++++	+++++
J. C.....	IIa	Sputum	11th	Rec'd	10^{-4}	—	++
R. E.....	IIa*	Lung	1st	Rec'd	10^{-5}	++	—
M. L. B.....	IIa*	Lung	4th	Rec'd	10^{-4}	+++	+++++
R. McN.....	IIa	Sputum	7th	Died	10^{-4}	—	+++++
Y. B.....	IIa	Blood	3rd	Rec'd	10^{-4}	+++++	+++++
H. F. L.....	IIa*	Sputum	5th	Rec'd	10^{-4}	—	—
F.....	IIa	Sputum	2nd	Rec'd	10^{-4}	+	+
H. C.....	III	Lung	1st	Rec'd	10^{-5}	+++	++
V. C.....	III	Blood	3rd	Died	10^{-4}	—	—
A. Z.....	III	Lung	3rd	Rec'd	10^{-3}	—	—
K. H.....	III	Sputum	2nd	Rec'd	10^{-5}	—	—
J. B.....	IV	Sputum	5th	Rec'd	10^{-5}	+++	++
S. B.....	IV	Sputum	4th	Rec'd	10^{-3}	+++++	+++++
F. V.....	IV	Lung	6th	Rec'd	10^{-3}	—	—
H. F. P.....	IV	Blood	3rd	Died	10^{-4}	—	—
E. J.....	IV	Sputum	1st	Rec'd	10^{-5}	—	—

—, not done.

* Type V Cooper.

mixtures. Those able to grow in the serum and leucocytes in quantities as small as 10^{-5} were highly lethal for mice, killing in amounts of

10^{-7} of the standard suspension; while the strains which were killed in amounts of 10^{-3} of the standard suspension were of decidedly lower mouse virulence.²

Tests for the opsonic and agglutinative action of normal pooled human serum against these strains showed much more marked individual variation than did the pneumococcal. While all those tested were agglutinated and phagocyted under the influence of the normal serum, the degree to which these actions occurred differed considerably with the several strains. These fluctuations might be accounted for, at least to a certain degree, by the fact that the technique for opsonic and agglutinative effect is much less susceptible to quantitative measure than is the pneumococcal test.

Individual Variations

When the serum and leucocytes from a single individual were tested with several types of pneumococci, striking variations in pneumococcal action against different organisms were often observed (Table III). This ranged from marked effect on one organism to none against another. Table IV gives a summary of the tests in 15 normal individuals using three to four types in each case.³ Two experiments included three different individuals each, the first six in the table, tested against all four types at the same time. Tests on individuals T. B., R. G., and G. S. were also carried out in one experiment. The plus signs indicate degree of pneumococcal effect. + = killing of 10^{-7} of the standard suspension; ++ = 10^{-6} , etc. The letters following the signs designate the strain of organism employed. It will be noted (Table IV) that in certain instances, the serum and leucocytes of one individual show marked killing power for an organism against which the blood elements of another person are without any effect.

² This relationship did not seem to hold as closely when the tests were repeated months or a year following isolation. Certain strains, notably those of Type II, had lost much of their growth capacity in human serum-leucocyte mixtures but still retained their mouse virulence.

³ In the majority of these experiments, the sera from different persons were used with leucocytes from a single individual. This was possible because of the lack of any detectable individual variations in the activity of the leucocytes of normal human beings in the above tests.

These differences are of such a magnitude as to preclude their being due to errors in technique. Furthermore they were corroborated by

TABLE III

Variations in Pneumococcal Activity of a Normal Human Being's Serum and Leucocytes for Several Types of Pneumococci

Serum 0.2 cc. + leucocyte suspension 0.1 cc. + pneumococcus suspension 0.1 cc. + gelatin Locke's solution 0.1 cc.

Type	Amount of pneumococcus suspension	Growth as shown by methemoglobin formation at			Survival of pneumococci at 72 hours
		15 hrs.	46 hrs.	72 hrs.	
I	10^{-3}	+	+++	++++	+
	10^{-4}	0	0	0	0
	10^{-5}	0	0	0	0
	10^{-6}	0	0	0	0
	10^{-7}	0	0	0	0
IIa (V Cooper)	10^{-4}	++++			+
	10^{-5}	++++			+
	10^{-6}	++++			+
	10^{-7}	++++			+
II	10^{-4}	0	0	0	0
	10^{-5}	0	0	0	0
	10^{-6}	0	0	0	0
	10^{-7}	0	0	0	0
Controls with serum only					
I	10^{-7}	++++			+
IIa	10^{-7}	+	++++		+
II	10^{-7}	++++			+
Plates of 10^{-7} dilution of the pneumococcus suspensions					
I	No. of colonies		114		
IIa	" " "		46		
II	" " "		14		

repeated tests which gave essentially the same results. All persons tested possessed pneumococcus-killing blood properties of some degree for at least one type of organism while the majority showed them for

two or more.⁴ The only type for which every individual exhibited pneumococcal action was Type II.

It was now of interest to determine whether these varying individual responses to different pneumococci depended on the particular strain used or on the type. Accordingly the serum and leucocytes of five of

TABLE IV

Variations in Pneumococcal Activity of Serum and Leucocytes of Normal Individual Human Beings for Several Types of Pneumococci

Normal individual	Pneumococcal activity of blood for types			
	I	II	III	IIa (V Cooper)
M. A. C.	0 (El.)	++++ (Vo.)	+++++ (Ca.)	0 (En.)
W. R.	0 (El.)	++++ (Vo.)	++++ (Ca.)	0 (En.)
D.	0 (El.)	+++++ (Vo.)	+++ (Ca.)	++ (En.)
O. H. R.	+++ (El.)	++++ (Pa.)	0 (McT.)	+
E. S. L.	++++ (El.)	++++ (Pa.)	++ (McT.)	+++++ (En.)
H. V. S.	+	++ (Pa.)	0 (McT.)	0 (En.)
E. E. T.	0 (El.)	+++++ (Pa.)	+++ (McT.)	—
N.	++++ (El.)	—	—	+++++ (En.)
T. H.	++++ (St.)	++++ (Me.)	—	0 (En.)
T. B.	+++ (At.)	+++ (Vo.)	+++++ (Cl.)	—
R. G.	++ (At.)	+++++ (Vo.)	+++++ (Cl.)	—
				Group IV
G. S.	0 (At.)	+++++ (Vo.)	0 (Cl.)	0 (Ho.)
F. R.	+++ (At.)	+++++ (Vo.)	++ (Cl.)	—
T. E. F.	+++++ (At.)	+++ (Vo.)	+++++ (Zl.)	—
J. G.	+++++ (At.)	+++ (Vo.)	+++++ (Zl.)	—

+, killing of 10^{-7} of the standard suspension. ++, killing of 10^{-6} of the standard suspension. +++, killing of 10^{-5} of the standard suspension. Etc.

Letters in parentheses indicate strain of pneumococcus.

—, not done.

the above persons were tested with three separate strains of each type. Two of the individuals, E. S. L. and T. B., showed a well marked killing

⁴ In an earlier paper on natural immunity to pneumococci (4) human beings were included in the group of mammals relatively susceptible to pneumococcus infection. This erroneous conclusion resulted from the finding that the serum of the several human beings tested showed no opsonic action for the single strain of pneumococcus used, a virulent Type I.

action for all of the types used. The results recorded in Table V seem to indicate that type is the conditioning factor. While there occurred variations in degree of reaction between the different strains yet by and large these were less than the differences between types. However, certain strains showed more growth activity in the serum-leucocyte mixture than others. Two of these, R., a Type I and McT., a

TABLE V

Comparative Pneumococcal Power of Individuals' Serum and Leucocytes for Different Strains of Several Pneumococcus Types

Normal individual	Pneumococcus type	Pneumococcal activity for different strains					
M. A. C.	I	0	(El.)	0	(Ri.)	+	(As)
	II	++++	(Vo.)	++++	(Pa.)	+++	(Mc.)
	III	++++	(Ca.)	+++	(McT.)	++++	(Bi)
	IIa*	0	(En.)	+	(Br.)	+	(LV.)
W. R.	I	++	(El.)	0	(Ri.)	++	(As)
	II	+++++	(Vo.)	+++++	(Pa.)	+++	(Mc.)
	III	++++	(Ca.)	++	(McT.)	++++	(Bi)
	IIa*	0	(En.)	++	(Br.)	+	(LV.)
E. S. L.	I	++++	(El.)	+	(Ri.)	+++	(Bio)
	II	++++	(Vo.)	+++++	(Pa.)	++++	(Mc.)
	III	+++++	(Ca.)	+	(McT.)	+++++	(Bi)
	IIa*	+++	(En.)	++++	(Br.)	+++++	(LV.)
T. B.	I	+++++	(McC.)	++++	(Al.)	++	(As)
	II	+++	(Vo.)	+++++	(Pa.)	+++++	(Mc.)
	III	++++	(Cl.)	+++++	(Zl.)	++++	(Ha.)
G. S.	I	0	(McC.)	+	(Al.)	+	(As)
	II	+++	(Vo.)	++	(Pa.)	++	(Mc.)
	III	+	(Cl.)	0	(Zl.)	0	(Ha.)

* Type V Cooper.

Type III were consistently inhibited to a decidedly less degree than were the other members of the same type, which suggests that these strains were of higher human virulence.⁵

⁵ They were not included with the freshly isolated strains as they had been secured a number of months earlier.

DISCUSSION

The above study while comprising only a relatively small number of pneumococcus strains, affords no evidence than any one type of pneumococcus is of higher virulence for human beings than the others. In fact, the pneumococidal potency of the pooled normal human serum and leucocytes exhibits a surprising uniformity for the several types of pneumococci. Variations shown for different strains were not confined to any one type. Possibly the inclusion of a larger number of the members of Group IV would show that these organisms have, as a class, a lesser growth capacity in normal human serum and leucocytes than the so-called fixed types, although the recent reclassification of Group IV and Type II atypical pneumococci by Cooper, Edwards and Rosenstein (5) indicates that a considerable percentage of these organisms are to be considered as highly pathogenic as the members of Types I, II, III.

Clough's (6) findings that the serum of certain normal human beings showed mouse protective properties which differed in degree against the several types of pneumococci suggested that natural humoral anti-pneumococcus immunity, in man, is characterized by fairly wide individual variations. Our findings support this supposition and further indicate the nature of man's natural humoral immunity.

It is, of course, recognized that resistance to pneumococcus infection, particularly that of the lungs, is dependent upon factors both local and general in addition to the pneumococcus-killing power of the blood. But with the present lack of appropriate methods we have no means of estimating the functional activity of these other defense processes such as the eliminatory function of the lungs, the phagocytic activity of the fixed macrophages, etc.; of knowing which is the principal and which the secondary means of defense. However, in view of the studies on experimentally infected animals referred to earlier, in which a close correspondence was found to exist between resistance to pneumococcus infection and the pneumococidal power of their blood it seems fair to assume that the presence of this same property in the blood of human beings indicates their capacity to combat invasion by pneumococci in any part of the body to which the blood has free access.

In relation to the high mortality of lobar pneumonia caused by

Pneumococcus Type II it was surprising to find that the group of 15 normal individuals tested showed a more constant pneumococcal power for organisms of this type than for those of other types. That this result is not to be attributed to the employment of strains of relatively low human pathogenicity is indicated by the fact that two of them were isolated from the blood of fatally terminating cases, one a fulminating case dying within 48 hours. However, there is some evidence to suggest that the virulence of these Type II strains for human beings may have diminished before being employed in the tests with normal individuals. When first isolated the two blood strains were killed by the pooled serum-leucocyte mixtures in quantities of the suspension only to 10^{-5} . Two months later they were killed by the blood of normal individual human beings in quantities of 10^{-4} or 10^{-3} yet their mouse virulence appeared to be unaltered.

That an individual should exhibit such marked variations in pneumococcal activity for the different types of pneumococci is of particular interest in view of our previous findings in other pneumococcus resistant mammals. While an occasional deviation was observed in tests on dogs and cats it was slight in degree compared to those found in this study. The reactions of the human serum-leucocyte mixtures are much more comparable in this respect to those of a pneumococcus-susceptible animal such as the rabbit which shows marked individual fluctuations both in resistance to pneumococcus infection and in concentration of natural humoral immune bodies for different pneumococcus types. Whether or not these apparently specific reactions of the human blood elements depend on the presence of distinct opsonins for the several types of pneumococci we have not determined. Our knowledge of the nature of this reaction suggests that such is the case (4). Sia's recent finding of specific opsonins and mouse protective substances in normal pig serum against *Pneumococcus* Types I, II and III lends support to this view (7, 8).

There has appeared as this manuscript was about to be sent for publication a paper by Ward (9) dealing with the pneumococcal power of the defibrinated blood of normal persons for different types of pneumococci. While Ward's technique varied considerably from ours and no mention is made of the source or time of isolation of the pneumococcus cultures used, his findings of a wide variability in blood

pneumococcal activity between several individuals for a given type and in a single person for the three types of pneumococci agree essentially with our observations on these points.

SUMMARY

With a view to obtaining information as to the virulence of pneumococci for human beings a study was made of the pneumococcal action of normal human serum-leucocyte mixtures for freshly isolated strains of pathogenic pneumococci. It was found that human beings as a group showed well marked pneumococcus destroying power in their blood for all types of organisms studied. Individuals, however, exhibited wide variations in their reactions against the different types. These ranged from marked killing effect for one type of pneumococcus to none or slight against another. While reactions against different strains within the type often varied considerably this difference was less, on the whole, than that between types. An interpretation of these findings in the light of previous animal experiments in which actual determination of resistance to pneumococcus infection was made leads to the inference that human beings in general possess a considerable degree of natural immunity to all types of pneumococci but that individuals may be relatively susceptible to one or more types and at the same time resistant to others; also that pathogenic strains of pneumococci vary much in their virulence for man.

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THE ORIGIN AND FATE OF TWO TYPES OF MULTINUCLEATED GIANT CELLS IN THE CIRCULATING BLOOD

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PLATES 10 AND 11

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In the course of studies in the experimental production of giant cells in the tissues, reported by Doan, Sabin and Forkner (1), the writer discovered a procedure by which giant cells could be made to appear in the blood stream. This new material gave valuable evidence concerning the formation of giant cells, more especially of those of the so called foreign body and epithelioid types.

As far as can be ascertained, no one has hitherto reported a method to induce an appearance of giant cells in the blood, and no such cells have been found there, except megakaryocytes, which were reported by Oelhafen (2) and Naegeli (3) in 1914, by Minot (4) in 1922, by Sabin (5) in 1923, and by others. The usual condition in which they have been found is myelogenous leucemia.

Haythorn (6), in a recent review, has classified giant cells as follows: (a) Langhans' giant cells, (b) foreign body giant cells, (c) osteoclasts, (d) megakaryocytes, (e) muscle giant cells, (f) giant cells of nervous tissue, and (g) true tumor giant cells. It is relative to the first two of these types that this paper is concerned.

As early as 1868 Langhans (7), working with fresh tissues, demonstrated large multinucleated cells obtained from tuberculous lesions. For the most part the nuclei were arranged around the periphery of the cell, were usually round or oval, had sharp outlines and generally contained nucleoli. He stated that the protoplasm of such a giant cell was pale, homogeneous, or finely granular, with the center usually clear. This type of cell has since been designated the "Langhans' giant cell." However, some confusion has arisen because he also described another type of giant cell which had nuclei distributed throughout the cytoplasm.

For reasons given by Doan, Sabin and Forkner (1), the term "*epithelioid giant cell*" has been proposed to replace the often misunderstood term of Langhans' giant cell. This concept of the "epithelioid giant cell" is based on new information obtained through the methods of studying living cells with supravital (Simpson, 8)

(Sabin, 5) and vital stains. It was first demonstrated by Sabin, Doan and Cunningham (9) that the epithelioid cell is derived from the monocyte. The supravital technique showed that in the monocyte, surrounding its centrosphere, is a characteristic rosette of vacuoles, stainable in the living state with neutral red. An accentuation of this normal structure of the monocyte produces an epithelioid cell. Cunningham, Sabin, Sugiyama, and Kindwall (10) then confirmed the findings of other investigators that the Langhans' giant cell is a multinucleated epithelioid cell.

In general there have been two opinions concerning the types of cells under discussion. One group of investigators, among them Ziegler (11), Kockel (12), Hektoen (13), Maximow (14), and Medlar (15), believe that the Langhans' giant cell is merely a foreign body giant cell occurring in tuberculosis. Krückmann (16) studied giant cells in tuberculosis, about parasites and foreign materials, and in tumors. He concluded that microscopic means were not available by which foreign body giant cells and Langhans' giant cells could be distinguished. Langhans (7), Jacobson (17), Lubarsch (18) and others, however, believe that the giant cell of tuberculous tissue can be distinguished from all other kinds.

Concerning the mode of origin of the foreign body and epithelioid giant cells there has been some disagreement. Weigert (19), Baumgarten (20), Bakács (21), Lubarsch (18), and others were of the opinion that giant cells of tuberculous lesions were formed by continued nuclear division. Krauss (22), Mallory (23) and Wells (24) are among those who favor fusion of individual cells as the explanation for the formation of the types of cells under discussion. Lewis and Webster (25) in 1921 believed that epithelioid giant cells were formed by amitosis of the nucleus, but a later paper by Lewis (26) takes the opposite view, that they are formed by fusion of epithelioid cells.

Materials and Methods

The experiments reported in this communication have been carried out with the use of the methods of supravital and vital staining. Because of some recent minor modifications in the methods used in Dr. Sabin's laboratory, the solutions of dyes employed and their method of preparation will be described in detail.

We are now using six different solutions for our routine work. Solution 1 is prepared by dissolving 125 mg. of vital neutral red (Grübler) in 50 cc. of neutral absolute ethyl alcohol. This makes a 0.25 per cent solution which is saturated. It may be stated here that neutral red (Ehrlich) certified for use in vital staining as prepared by the National Aniline and Chemical Company, New York, is a thoroughly satisfactory dye which can be substituted for neutral red (Grübler). Solution 2 is prepared by dissolving 125 mg. of vital Janus green (National Aniline or Grübler) in 62.5 cc. of neutral absolute ethyl alcohol, thus making a 0.20 per

cent solution which likewise is saturated. By adding 50 drops of Solution 1 to 10 cc. of neutral absolute ethyl alcohol, we prepare Solution 3. Solution 4, a mixture of neutral red and Janus green, is made by adding 2 drops of Solution 2 to 3 cc. of Solution 3.

For the study of blood or other tissues, where cells to be stained are relatively few in number, we prepare slides in the manner described by Sabin (5). This is accomplished as follows: (a) by means of a pipette, flood the upper surface of a slide chemically clean with Solution 4; (b) drain excess of dye back into bottle by holding slide in an upright position; (c) absorb the excess of dye on the dependent edge of the slide with blotting material; (d) evaporate the remaining alcohol on the slide by immediately holding it over a piece of wire gauze under which a gas flame is burning. There is no objection to burning the excess of alcohol from the slide. The stained surface of the slide is then marked with a wax pencil and the slides are stored for use. Some investigators in the past have experienced difficulties in obtaining an even distribution of the dye over the slide, particularly when there is a high degree of humidity in the atmosphere. Preparing the slides as described, in artificially dried air, obviates these troubles, leaving a thin, even film of the dye.

For study of bone marrow, lymph nodes, or other tissues where there is an abundance of colorless cells to be stained, two more solutions are required. Solution 5 is prepared by adding 150 drops of Solution 1 to 10 cc. of neutral absolute ethyl alcohol. Solution 6, a mixture of neutral red and Janus green, is made by adding 8 drops of Solution 2 to 3 cc. of Solution 5. Thus Solution 6 contains approximately three times the amount of the dyes as Solution 4. Slides are prepared with Solution 6 in precisely the same manner as with Solution 4. The latter solutions, 4 and 6, being mixtures of neutral red and Janus green, deteriorate after about 24 to 48 hours and must be freshly prepared each time they are needed. The remaining solutions are stable and are best kept in glass stoppered bottles in a cool, dark place. Care must be exercised to make sure that the pipettes and bottles used are free of alkali. If an excess of alkali is present, the neutral red solution will become a muddy yellow color.

Blood films in these supravital studies are prepared by obtaining a drop of blood on a clean coverslip and letting it fall gently on the slide prepared from Solution 4. The film is quickly rimmed with vaseline (salvoline) and in the course of from 3 to 5 minutes is ready for study. The films are best studied in a constant temperature box at 38° or 39°C. For the study of the cells of organs such as the omentum or subcutaneous tissues, thin films of the intact tissue may be spread over slides prepared with Solution 6. If the organs are too dense or too thick to be spread intact over a slide, the cut surface of the fresh tissue, for example, lung, lymph nodes, etc., is scraped with a sharp scalpel and the tissue accumulating on the knife blade is placed on a slide prepared from Solution 6. If the tissue is very dry a drop of plain normal salt solution or normal salt solution moderately colored with neutral red may be added to suspend the cells. A coverslip is then applied as above and is rimmed with vaseline. Material prepared in this way provides a thin enough film so that the individual living cells can be studied. For our

purposes, tissues fixed in Helley's fluid, embedded in paraffin, and stained by the usual methods were used to supplement the supravital studies.

The fundamental experiments dealing with giant cells were carried out on the blood and tissues of rabbits which had been injected with various substances, namely, agar, lycopodium spores, paraffin, olive oil, mineral oil, living tubercle bacilli, and certain lipoid fractions isolated from tubercle bacilli. These latter substances were obtained from Dr. R. J. Anderson of the Sterling Chemical Laboratories of Yale University, through the Research Committee of the National Tuberculosis Association. A full account of these reactions has been presented by Doan, Sabin and Forkner (1). None of the substances studied produced giant cells in the circulating blood, except agar. Consequently the development of this special phase of the giant cell study is here presented separately.

Experimental Data

The early experiments in which relatively small amounts of agar were introduced into the tissues produced no significant changes either quantitative or qualitative in the cells of the blood. However, since the local tissue response was so profound such large numbers of monocytes and giant cells having been produced, the possibility presented itself that a larger and more disseminated foreign body reaction might carry over the tissue response to the blood. This theory seemed to have merit since it was known from experience in this laboratory that certain diseases, notably tuberculosis, are often associated with the finding of pathologic tissue elements, epithelioid cells, in the circulating blood.

Accordingly, an animal, R 790, was injected, on February 25, 1929, with plain sterile agar in many areas under the skin and intraperitoneally.

A total of 40 cc. of the agar at pH 7.4 was introduced. Incidentally this animal was given frequent intravenous injections of trypan blue in order that the reactions of the giant cells of the tissues to this vital dye might be studied. Their responses are discussed elsewhere (1). Let it suffice to say that the trypan blue did not materially influence the blood picture in this animal, except that it may have been responsible for the increased number of macrophages.

The blood had been examined prior to the injections, but was not studied again

until 1 week later (March 4). At this time there was a slight reduction of the hemoglobin and number of red corpuscles. The total white blood cell count was not changed. Monocytes were distinctly high (21 per cent) and the lymphocytes were lower than they had been in the control period. The blood platelets were clustered in large masses. Some individual blood plates were larger than normal. The platelets were undoubtedly greatly increased in number. The blood contained 11 per cent of clasmotocytes (macrophages) which were usually large cells with single, centrally placed nuclei. Their cytoplasm contained debris of various shades of color and various sizes and shapes. The presence of the large number of these cells on this occasion may be explained as the result of the injection of trypan blue. Such phenomena have been studied and reported by Simpson (8). The character of the monocytes on this and subsequent days was of great interest. Many of them were much larger than normal, possessed round or oval nuclei, often with two or three nucleoli, and each cell contained a rosette of exceedingly fine uniform neutral red bodies. In some instances the rosettes were small, whereas in others they were hypertrophied, making a stimulated monocyte which is a transition between a normal monocyte and an epithelioid cell. These forms are entirely similar to the developing monocytes which Forkner (27) has described and illustrated as normal elements in peripheral lymph nodes of rabbits. Another interesting change was noted in the polymorphonuclear neutrophils. Many of these cells had a decreased number of specific granules and contained more neutral red bodies than are normally present. These neutral red bodies normally are round or globular whereas after agar injections the same bodies are present and in addition many larger, often elongated, non-refractive vacuoles or granules.

Besides these changes in the usual blood cells, typical multinucleated giant cells could be found in every smear on this and subsequent days until the animal was autopsied on March 18, 1929, 3 weeks after the agar injection. The photograph of one of these cells is illustrated in Fig. 11. This cell had about 25 nuclei scattered through all parts of the cytoplasm. There was an area near the center of the cell which was probably agar. It was a homogeneous amorphous material which had a purple tint. It had apparently taken on some of the trypan blue as the agar masses in the tissue had done. The nuclei near the periphery tended to be oval, whereas the others were round in shape. Individual nuclei were quite uniform in size and were somewhat larger than red blood corpuscles. Some of them contained nucleoli. The cytoplasm contained scattered, irregular masses of material staining all shades of red, yellow, and brown. There was no pattern to the arrangement of the material stained with neutral red.

Fig. 9 represents a cell from the peripheral blood of this animal. A coarse mass of agar is seen to lie across the center of the cell and on either side are segregation bodies entirely similar to those encountered in epithelioid cells and monocytes. One can, in fact, make out two areas in which the arrangement of the vacuoles suggests rosettes of neutral red bodies in the upper part of this cell, indicating a possible fusion of two or more mononuclear or polynuclear cells around the large

mass of agar. This last cell (Fig. 9) is difficult to classify in one or the other group, but on the whole it is more like a foreign body giant cell.

Another interesting group of cells from Rabbit R 790, shown in Fig. 4, illustrates how the giant cells may be formed or how they may be disintegrated. In this figure are three stimulated monocytes which have fused together about a blue mass of agar. When first seen this group had an almost spherical cell membrane surrounding the three cells and the agar. They were observed in the warm box for about 2 hours and were seen to separate into three distinct living cells with only a thread of cytoplasm connecting them. The agar was left free between them. This phenomenon may represent the fate of foreign body giant cells by disintegration into separate uninuclear cells. Other instances have been observed where giant cells of the foreign body type could be seen to be composed of many individual monocytes with other monocytes or epithelioid types nearby, apparently fusing to form a giant cell (Figs. 12 and 13). It would appear then that this is evidence similar to that recorded by Hektoen (13). He studied healing, non-degenerative tuberculous tissue of the brain and found that many giant cells are disintegrated into mononuclear cells which retain their function. It is not clear from his text whether the giant cells which he observed as separating into mononuclear elements were according to the classification here employed "epithelioid giant cells" or foreign body giant cells. I have found no evidence for this phenomenon in "epithelioid giant cells." Later Hektoen (28) supported his former view by observing the same phenomenon in the fate of the giant cells which form in the absorption of coagulated blood serum, in the anterior chamber of the rabbit's eye. In these latter experiments it seems that he was probably dealing with the foreign body type of giant cells.

Rabbit R 579 was injected with agar as the previous animal had been, but no dye was introduced.

A preliminary study of the blood before the injection showed a normal formula. There were 7,600 white blood cells and 5,250,000 red blood cells per cubic millimeter. There were 11 per cent monocytes. 2 days after the injection of agar, the monocytes rose to 20 per cent and increased in total number from 836 to 1,580 per cubic millimeter. 6 days after the injection, monocytes became more numerous than any other cell, increasing to 49 per cent or 5,120 cells per cubic millimeter. A chart (Chart 1) makes this reaction clear. In this experiment, there was a reciprocal relationship between the numbers of monocytes and lymphocytes. As in the preceding experiment, many of the monocytes were of the young type, larger than the adult cells, possessing round or oval nuclei with nucleoli and having an extraordinary abundance of delicate mitochondria. Some of the mature monocytes contained coarse irregular brownish bodies in their cytoplasm, which were probably pieces of agar undergoing digestion.

Giant cells were seen in the blood on the eighth and ninth days. On the eighth day enough were present to be recorded in the differential count on a percentage

basis; on the ninth day, only an occasional cell in an entire film, indicated on the chart by a + sign, was seen. One of these was drawn (Fig. 6). It is clearly of the foreign body type with scattered nuclei and abundant irregular masses of material in the cytoplasm, probably agar undergoing fragmentation and disintegration. The nuclei contained definite nucleoli. Mitochondria were present and easily seen near the periphery of the cell. Adjoining this cell but not fused with it is a monocyte containing the same kind of material as in the giant cell. It is apparent that the larger cell might easily be considered as representing a fusion of many of the individual monocytes. Another cell was drawn (Fig. 7) which has a central rosette and three peripheral nuclei. The material in the cytoplasm is

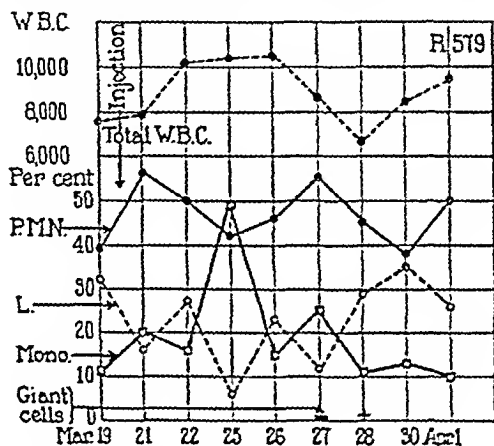


CHART 1. Graph of the white blood cells of Rabbit R 579. The arrow indicates the time at which 40 cc. of sterile plain agar was given in many subcutaneous areas and intraperitoneally. For giant cells the symbol + means the presence of an occasional giant cell in an entire preparation; the solid blocks indicate their percentages when sufficient to appear in the differential count.

not typical of an epithelioid cell. It looks much more like a small foreign body giant cell. It is impossible to say whether this giant cell has arisen by fusion of independent elements or has formed by division of the nuclei. Fig. 8 represents a small foreign body giant cell of the same type as Fig. 7, but containing much coarser bodies. The nuclei are flattened out against the cell wall. This cell, together with that shown in Fig. 7, illustrates that one can not always identify this type of giant cell by the peripheral arrangement of the nuclei. In order to differentiate them properly one must study all of the cellular organs. The presence of a central rosette of fine neutral red bodies aids materially and is probably the most important single factor in the separation of "epithelioid giant cells" or the so-called Langhans' cell. The lungs of this and the other animals were studied in

TABLE I
Changes in the Blood Cells as the Result of Agar Injections into the Tissues

Experi- ment	Date	White blood cells per cu. mm.	Red blood cells per cu. mm.	Neutrophils		Basophils		Eosinophils		Monocytes		Lymphocytes		Giant cells		Clasmato- cytes		Unclassified		Hemo- globin per cent
				Per cent	Per cu. mm.	Per cent	Per cu. mm.	Per cent	Per cu. mm.	Per cent	Per cu. mm.	Per cent	Per cu. mm.	Per cent	Per cu. mm.	Per cent	Per cu. mm.	Per cent	Per cu. mm.	
R 790	1/12/29	4,850	5,490,000	45	2,182	5	242	1	4811		53338		1,843							68
	1/14	6,400	5,800,000	30	1,920	14	896	2	128 7		44847		3,008							65
	2/25	6,250	6,200,000	56	3,500	8	500	1	63 7		43828		1,750							76
	2/25	Began frequent intravenous injections of trypan blue. Injected 40 cc. of agar intraperitoneally and subcutaneously.																		
	3/4	5,500	4,580,000	52	2,860	6	330		21		1,15510		550	+		11	605			57
	3/6	4,100	4,000,000	58	2,378	14	574	1	41 6		24618		738	+		3	123			56
	3/7	7,500	4,140,000	63	4,725	10	750	1	75 2		15022		1,650	+		1	75	1	75	55
	3/9	3,800	4,360,000	62	2,356	12	456	1	38 5		19019		722	++						49
	3/11	4,200	4,940,000	72	3,024	1	42	2	84 1		4223		966	+		1	42			56
	3/12	7,950	4,480,000	72	5,724	2	159	2	159 3		23821		1,669	+		1	83			61
R 579	3/15	8,300	3,790,000	68	5,644	18	1,494	1	83 4		732 8		664	+		2	145	2	145	53
	3/18	7,250	4,690,000	53	3,842	22	1,595		8		58013		942	+						62
	3/19	7,600	5,260,000	39	2,964	17	1,292	1	7611		83632		2,432							
	3/19	Injected in 6 subcutaneous areas intraperitoneally with 40 cc. of plain agar.																		
	3/21	7,900	4,840,000	56	4,424	5	395		20		1,58016		1,264					3	237	52
	3/22	10,200	4,540,000	50	5,100	4	408	2	20416		1,63227		2,754					1	102	55
	3/25	10,450	5,260,000	42	4,389	1	104		49		5,120 6		627					2	209	64
	3/26	10,550	5,210,000	46	4,853	10	1,055	4	42215		1,58223		2,426					2	211	59
	3/27	8,700	5,320,000	55	4,785	3	261		25		2,17512		1,044					4	348	64
	3/28	6,700	4,620,000	45	3,015	12	804	2	13411		73729		1,943	1	87			1	67	58
	3/30	8,450	4,520,000	38	3,211	13	1,098	1	8513		1,09835		2,957	++						
	4/1	9,500	4,790,000	50	4,750	12	1,140	2	19010		95026		2,470	++						55

[illegible]

TABLE I—*Continued*

Experiment	Date	White blood cells per cu. mm.	Red blood cells per cu. mm.	Neutrophils		Eosinophils		Monocytes		Lymphocytes		Giant cells		Clasmato-cytes		Unclassified		Hemo-globin per cent
				Per cent	Per cu. mm.	Per cent	Per cu. mm.	Per cent	Per cu. mm.	Per cent	Per cu. mm.	Per cent	Per cu. mm.	Per cent	Per cu. mm.	Per cent	Per cu. mm.	
R 939	5/20	8,450	4,610,000	49	4,140	4	338									2	169	
	5/20	Injected intraperitoneally and subcutaneously with about 40 cc. sterile plain agar containing 10 cc. of India ink.																
	5/21	26,100	3,690,000	76	19,836	2	522	3	783	14	3,654	5	1,305					
	5/22	18,850	3,810,000	60	11,310					25	4,712	14	2,639			1	188	
	5/23	10,300	4,490,000	65	6,695	1	103			27	2,781	7	721					
	5/24	14,850	4,680,000	50	7,425	4	594			23	3,415	19	2,821			1	148	
	5/27	6,750	4,620,000	47	3,172	3	202			24	1,620	22	1,485					
	5/29	7,650	4,330,000	67	5,125	2	153			11	841	20	1,530					
	5/31	6,500	4,640,000	65	4,225	2	130			8	520	22	1,430					
	6/3	10,100	4,910,000	80	8,080	1	101			3	195		1,616			1	101	
	6/5	8,750	4,200,000	92	8,050					2	202	16				1	87	
	6/6	8,150	4,930,000	86	7,009	8	652			4	350	3	262					
										2	163	4	326					

sections. Numerous giant cells were easily seen in the smaller capillaries. Fig. 14 illustrates one of the giant cells in the lung of this animal.

Rabbit R 580 received the same type of injections as R 790.

Trypan blue (1 per cent solution in distilled water) was given intravenously or intraperitoneally in 4 to 10 cc. doses every second day and in addition the animal re-

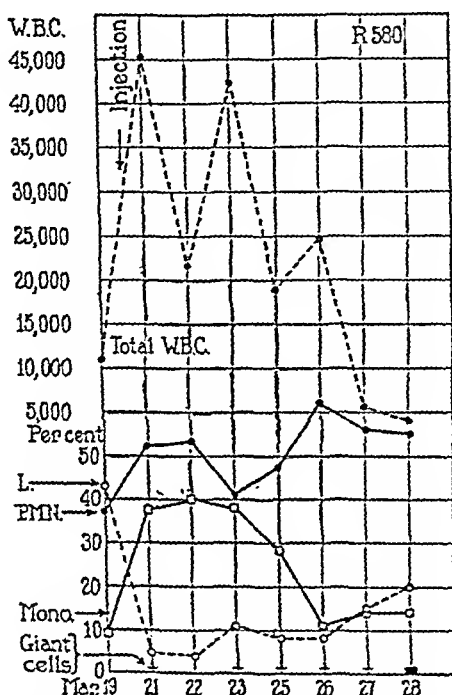


CHART 2. Graph of the white blood cells of Rabbit R 580. The arrow indicates the time at which 40 cc. of sterile plain agar was introduced subcutaneously and intraperitoneally. At this point injections of trypan blue were begun and were continued on every second day.

ceived plain agar into many areas of the subcutaneous tissue and peritoneal cavity. Prior to the injections the cells of the blood were entirely normal (Table I, Chart 2). There were 11,000 leucocytes per cubic millimeter. However, on the day following, the total leucocyte count rose to 45,500 cells per cubic millimeter—an increase of over 400 per cent. This rise was chiefly brought about by the monocytic strain of cells. The neutrophils also increased somewhat, but the lymphocyte curve descended, their number being represented by only 4 per cent of the total white

blood cells. At the height of the reaction there were over 17,000 monocytes in each cubic millimeter of blood. Many of these monocytes were of the young type previously described. Some of the monocytes and neutrophils appeared to contain fragments of agar. Many of the neutrophils had lost a considerable portion of their specific granules. There was a tendency for the leucocytes to be clumped in large masses. Some of the young monocytes contained two oval nuclei.

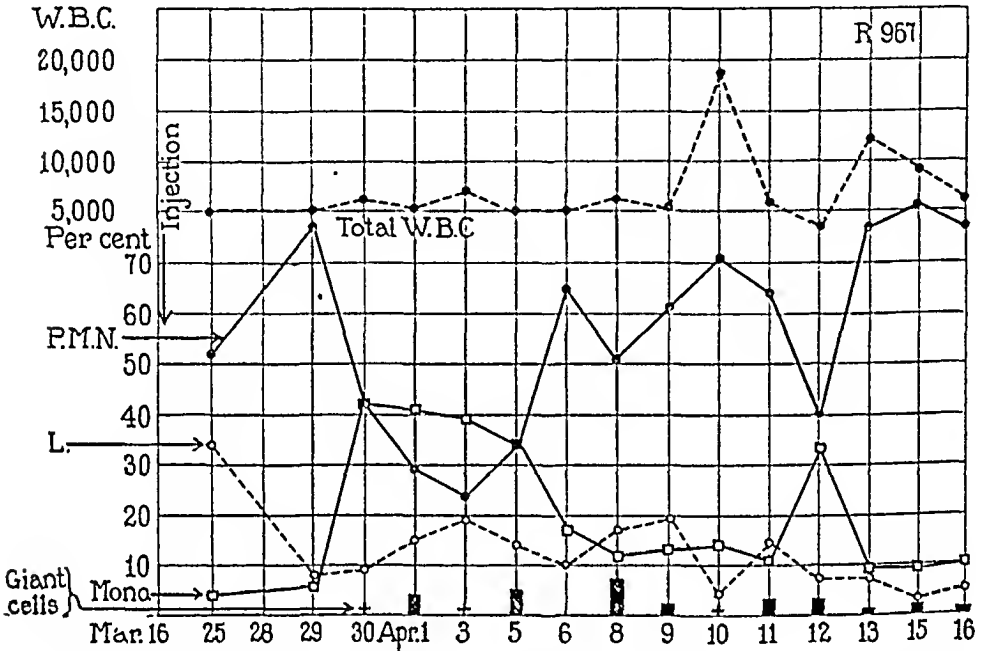


CHART 3. Graph of the white blood cells of Rabbit R 967. The first arrow shows the time at which the rabbit received 1 mg. of avirulent tubercle bacilli intravenously. This presumably did not modify any of the changes found in the chart. The second arrow indicates the time at which 40 cc. of sterile plain agar was administered subcutaneously and intraperitoneally. At this time also injections of trypan blue were begun and they were continued every second day.

2 days after the agar injection, the total leucocyte count was 21,900 per cubic millimeter, but the percentage of monocytes was even higher (40 per cent). The accompanying table and chart (Table I, Chart 2) will make these changes clear. 3 days after the beginning of the experiment the total white blood cell count was again elevated to 42,850 cells per cubic millimeter, but there were no significant changes in the percentages of cells except a rise of lymphocytes from 4 to 11 per cent. Giant cells of both types began to appear in the blood and could be found in practically every smear thereafter. 6 days after the injection, a mild leucopenia set in with the monocytes constituting about 14 per cent of the cells.

Rabbit R 967 was interesting because it showed giant cells in large numbers in the blood stream.

On March 16, 1928, the animal received an intravenous injection of 1 mg. of avirulent tubercle bacilli which did not produce progressive tuberculosis and which presumably caused no significant changes in the blood picture to be described. The blood picture was normal on March 25 when intravenous injections of trypan blue were begun and continued every second day for nine doses. This may account for the presence from time to time of from 3 to 5 per cent of clasmotocytes (macrophages) in the blood. The animal was injected with plain agar in a number of areas on March 28. The chart and table (Table I, Chart 3) show the changes in the total number and percentage values of the cells. The chief result of the injection on the blood was a rather striking and prolonged rise in monocytes together with a maintained decrease in the percentage and absolute number of lymphocytes.

This animal differed from the preceding one in that there was no sharp rise in total leucocytes within a short time after the injection. However, there did occur the marked elevation of percentage of monocytes. This increase was found on the second day and persisted for 1 week during which time the monocytes ranged from 34 to 42 per cent of the circulating white blood cells. During the next 2 weeks monocytes remained abundant, varying between 9 and 33 per cent. Giant cells occurred in the blood 2 days after the agar injection and were found in every film of blood until autopsy 17 days later. They frequently were so numerous as to constitute 2 or 3 per cent of the total number of leucocytes and on one occasion reached as high as 7 per cent. It was not uncommon to find two in a single high power field of the microscope. Most of the "epithelioid giant cells" contained from 2 to 7 nuclei (Fig. 15) but an occasional one had from 10 to 14 nuclei; the foreign body giant cells, on the other hand, contained from 2 to 50 nuclei, many of them having from 15 to 30.

Fig. 2 illustrates what might be called a mononuclear giant cell of the blood. It measured 26.7 by 31.4 microns, about twice the size of an ordinary monocyte. It had a large indented nucleus with a nucleolus, a definite rosette of fine neutral red bodies, together with numberless delicate mitochondria. This cell would fall into the monocyte group and would be further qualified by saying that it is a young stimulated form. If the rosette were slightly larger we would call it an epithelioid cell. 4 days after the injection, when monocytes constituted 41 per cent of the white blood cells, this type of cell, the young, large, stimulated form, was very abundant. Cells entirely identical with this except for the presence of 2, 3, or more nuclei were frequently found. Fig. 3 illustrates one of these epithelioid giant cells with 9 nuclei. All intermediate stages between the cells represented by Figs. 2 and 3 could readily be demonstrated in the blood.

At first the mechanism by which the giant cells reached the peripheral veins was not understood for some of these cells were more than

50 microns in their smallest diameters. It is difficult to conceive how these cells could find their way through the capillaries of the lungs and then into the systemic circulation. Figs. 5 and 10 illustrate how this phenomenon is accomplished. The cell in Fig. 10 was taken from the peripheral ear vein of Rabbit R 967 and shows that these giant cells are not rigid but that they may become elongated to such an extent as to pass through very small capillaries. Fig. 5 was from the

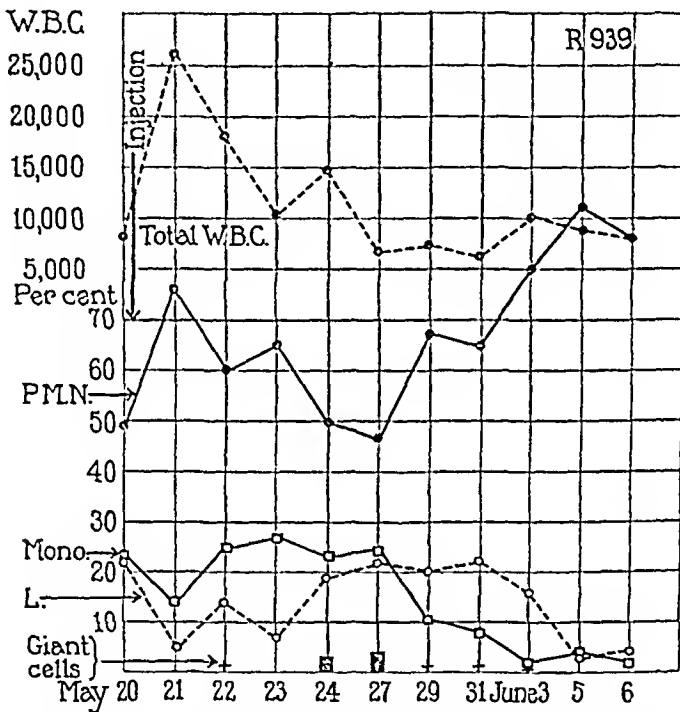


CHART 4. Graph of the white blood cells of Rabbit R 939. The arrow indicates the time at which 40 cc. of sterile plain agar was injected into the subcutaneous tissues and into the peritoneal cavity.

central artery of the ear of Rabbit R 939 and illustrates the apparent ease with which the cells may make their way through the capillary bed.

The next experiment which was tried was to mark the agar in some way in order that it might be identified when it was transported within cells and to distant parts of the body. This was carried out by adding to the 40 cc. of agar to be injected 10 cc. of Higgins' India ink. This was mixed carefully and made an intensely black preparation.

Rabbit R 939 was chosen for the experiment. The count prior to the injection showed the animal to have 23 per cent, an abnormally high value, of monocytes (Table I, Chart 4). On the day following the injection, the total leucocytes rose to 26,100 cells per cubic millimeter. The monocytes rose and remained high for several days. Giant cells of both types appeared in very small numbers in the blood on the second day and were found on several occasions over a period of 2 weeks. Occasionally a few granules of carbon could be seen in the giant cells, but not as much as was expected. Fig. 1 shows an epithelioid giant cell taken from the arterial blood, which has several definite fragments of agar containing carbon. The autopsy on this animal demonstrated that the carbon had not been as evenly distributed in the agar as was expected. The giant cells immediately adjacent to the ink deposits contained it in great abundance, but most held relatively little.

Many more animals were subjected to similar experiments with agar and with other substances. It was not possible to induce regularly an appearance of cells in the blood with any of the substances used except agar. In one animal which had been injected with large amounts of lycopodium spores, one giant cell was found in the blood on a single occasion. This finding could not be repeated.

DISCUSSION

These studies on the peripheral blood have made it possible to form definite ideas about the origin and development of "epithelioid giant cells" and of foreign body giant cells. It becomes necessary to consider these elements histologically as two groups of cells which have a common origin from monocytes. Under the conditions of these experiments it appears that the great majority of foreign body giant cells are formed by the fusion of monocytes, and that the majority of "epithelioid giant cells" are formed by amitotic division of the nucleus or nuclei of epithelioid cells which latter elements are monocytes of large size, with hypertrophied rosettes. The results of the studies would thus confirm the theories of both schools of pathologists, of those who consider that giant cells are the products of fusion, and those who maintain that they form by division of the nuclei. The difference of opinion on this point has resulted doubtless from the failure to recognize that in almost every tissue reaction which produces giant cells, both types are present, but that their relative numbers assume different proportions according to the character of the

substances which excite the response. No evidence has been observed in these studies to support the theory that epithelioid giant cells are formed by the fusion of individual cells. On the other hand, many epithelioid cells and "epithelioid giant cells" with partially divided nuclei, and others which appeared as though the nuclei had recently divided amitotically have been seen.

Regarding the fate of giant cells, no definite conclusions are possible but these studies lend support to the conclusion of Hektoen (28) that foreign body giant cells may separate into their constituent elements. No evidence is available that "epithelioid giant cells" undergo a similar process.

The occurrence of the two types of giant cells in the circulating blood may be explained in either of two ways. They may be formed in the blood vessels in the neighborhood of the foreign body reaction, or they may form extravascularly and migrate into the vessels. The latter explanation would appear more tenable, for giant cells are to some extent motile, and can become so elongated that passage through the capillary walls should be entirely possible. Furthermore, it would be difficult to explain the presence of large pieces of agar in the cells, as shown in Figs. 4 and 9, by any other hypothesis than that they have been carried within cells from the tissues into the blood.

SUMMARY AND CONCLUSIONS

1. It has been demonstrated that giant cells of the foreign body and epithelioid types can be induced to appear in the blood stream.
2. Evidence has been presented which indicates that foreign body giant cells are primarily formed by fusion of monocytes and that the fate of these giant cells is accomplished, at least in some instances, by a separation into the constituent elements.
3. Further evidence has been presented which lends support to the hypothesis that "epithelioid giant cells" reach their stage of evolution, not by fusion of monocytes, but by amitotic division of the nuclei of monocytes and epithelioid cells.
4. The presence of giant cells in the peripheral blood as the result of agar injections is almost invariably associated with, or preceded by a marked monocytosis in which the new monocytes are of large size and show evidence of immaturity.

5. Injections of agar into the tissues result in decreased absolute and percentage values of lymphocytes and a diminution of the specific granules in many of the polymorphonuclear leucocytes.

6. It would appear from these studies that a clear differentiation of "epithelioid giant cells" and "foreign body giant cells" in the blood is usually possible, but that on the other hand, a few cells may be present which have some of the characteristics of each type. These latter cells probably represent in their formation both a fusion of individual cells and an amitotic division of the nuclei of monocytes.

7. Clasmatoocytes or macrophages have in rare instances been seen to take part in the formation of foreign body giant cells. At least one instance has been noted of the fusion of a clasmatoocyte with several monocytes. No evidence is available to demonstrate that macrophages ever play a part in the formation of "epithelioid giant cells."

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EXPLANATION OF PLATES

PLATE 10

Drawings of cells from arterial and venous blood of rabbits after injections of agar; stained with neutral red and Janus green and drawn while still alive.

FIG. 1. An epithelioid giant cell from the peripheral arterial blood of Rabbit R 939. Small aggregations of carbon can be seen. The mitochondria are very abundant and are best seen at the periphery of the rosette. This cell measured 39.3 x 33 microns.

FIG. 2. A huge, young, stimulated monocyte from Rabbit R 967. It is almost as large as the cell in Fig. 1, but contains only a single nucleus with a large nucleolus. Thousands of delicate mitochondria are stained with Janus green and a typical dense rosette is stained with neutral red. Cells identical with this, except for the presence of two or more nuclei, could be found in practically every field. This cell measured 31.4 x 26.7 microns.

FIG. 3. Epithelioid giant cell from venous blood of Rabbit R 967, with nine nuclei. It has a unified central rosette, similar to those of Figs. 1 and 2. Size 36.1 x 33 microns.

FIG. 4. Three monocytes which have fused around a piece of agar in the blood of Rabbit R 790. This animal had received trypan blue intravenously on several occasions and the agar foci were stained in an identical manner, as illustrated in this figure. When first seen this cell was practically spherical, but after 2 hours the three constituent cells were almost entirely independent of each other. Size 35 microns at widest diameter.

FIG. 5. This huge giant cell was obtained from a peripheral artery of Rabbit R 939. It measured 107 microns in length. It illustrates very well that these cells are capable of changes in shape so great as to enable them to pass through the capillaries. A red blood corpuscle is shown to give an idea of their great sizes.

FIG. 6. A foreign body giant cell with many nuclei from the venous blood of Rabbit R 579. This cell and the monocyte nearby are filled with masses of irregular, fragmented material. This material is probably agar undergoing changes within the cell. Size 67.5 x 47.1 microns.

FIG. 7. A trinucleated cell containing the same general type of material as that in the cell of Fig. 6. The mitochondria have faded somewhat. This cell is also from the blood of Rabbit R 579. Size 21 microns in diameter.

FIG. 8. Another foreign body giant cell from the blood of the same animal. Size 28×17 microns.

FIG. 9. A foreign giant cell from the blood of Rabbit R 790. There is a large, lobulated mass of material in the center of the cell which is probably agar that has not as yet undergone very much change within the cell. In the upper left region of the cell two rosettes can be seen. Size 84×52.5 microns.

FIG. 10. Another elongated cell. This foreign body giant cell was from the blood of Rabbit R 967. Size 73.8 by 14 microns.

PLATE 11

Four photographs of living cells supravitaly stained with neutral red and Janus green, and one photograph (Fig. 14) from a section.

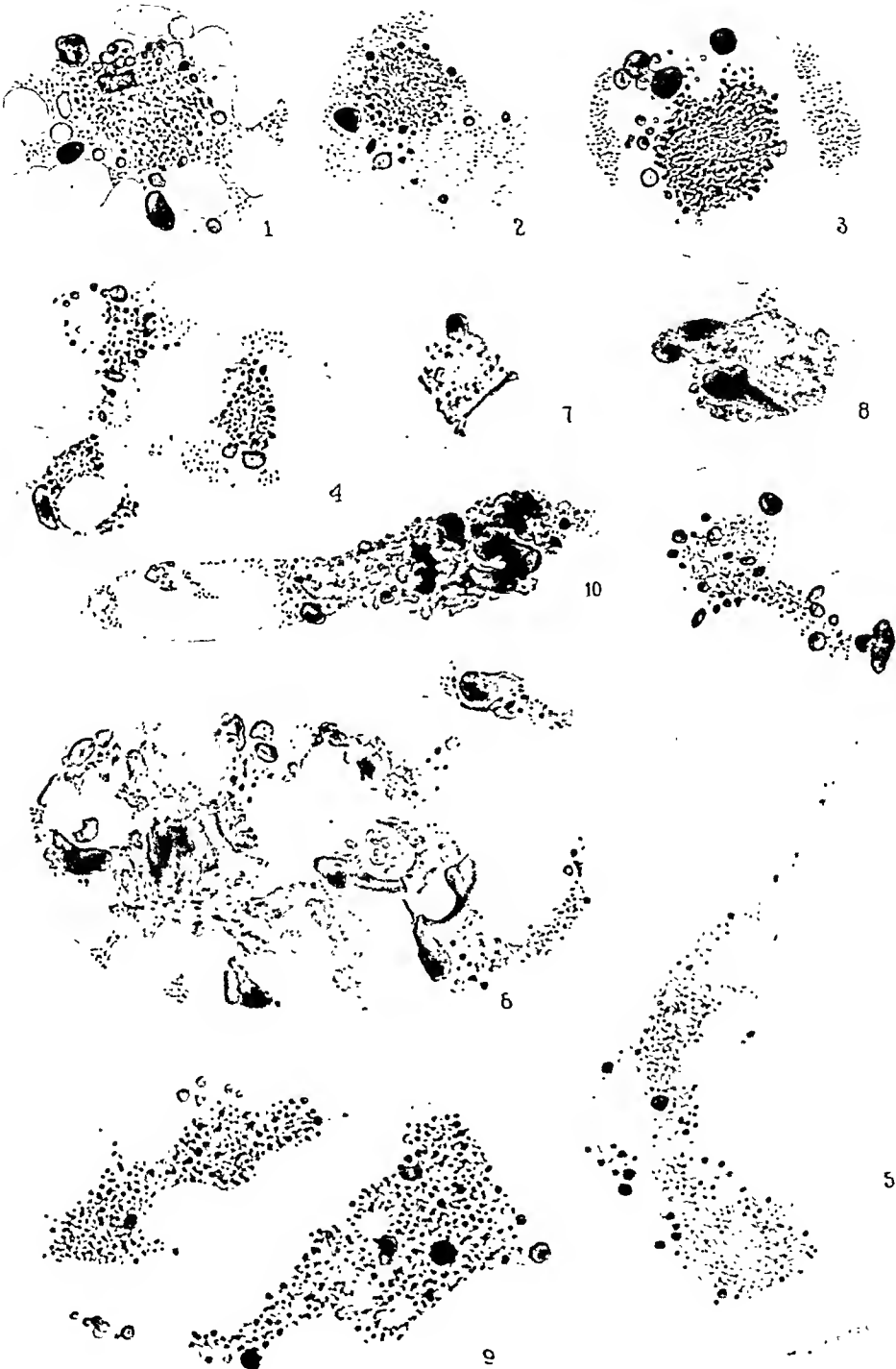
FIG. 11. Photograph of a large foreign body giant cell from the blood of Rabbit R 790. It has very many nuclei scattered throughout the cell and the cytoplasmic bodies are arranged in no definite pattern. Several monocytes can be seen on the surface of the cell, as if in process of fusion. Red blood corpuscles can be seen for comparison of size. Magnification $\times 575$. Stained with neutral red in supravital preparation.

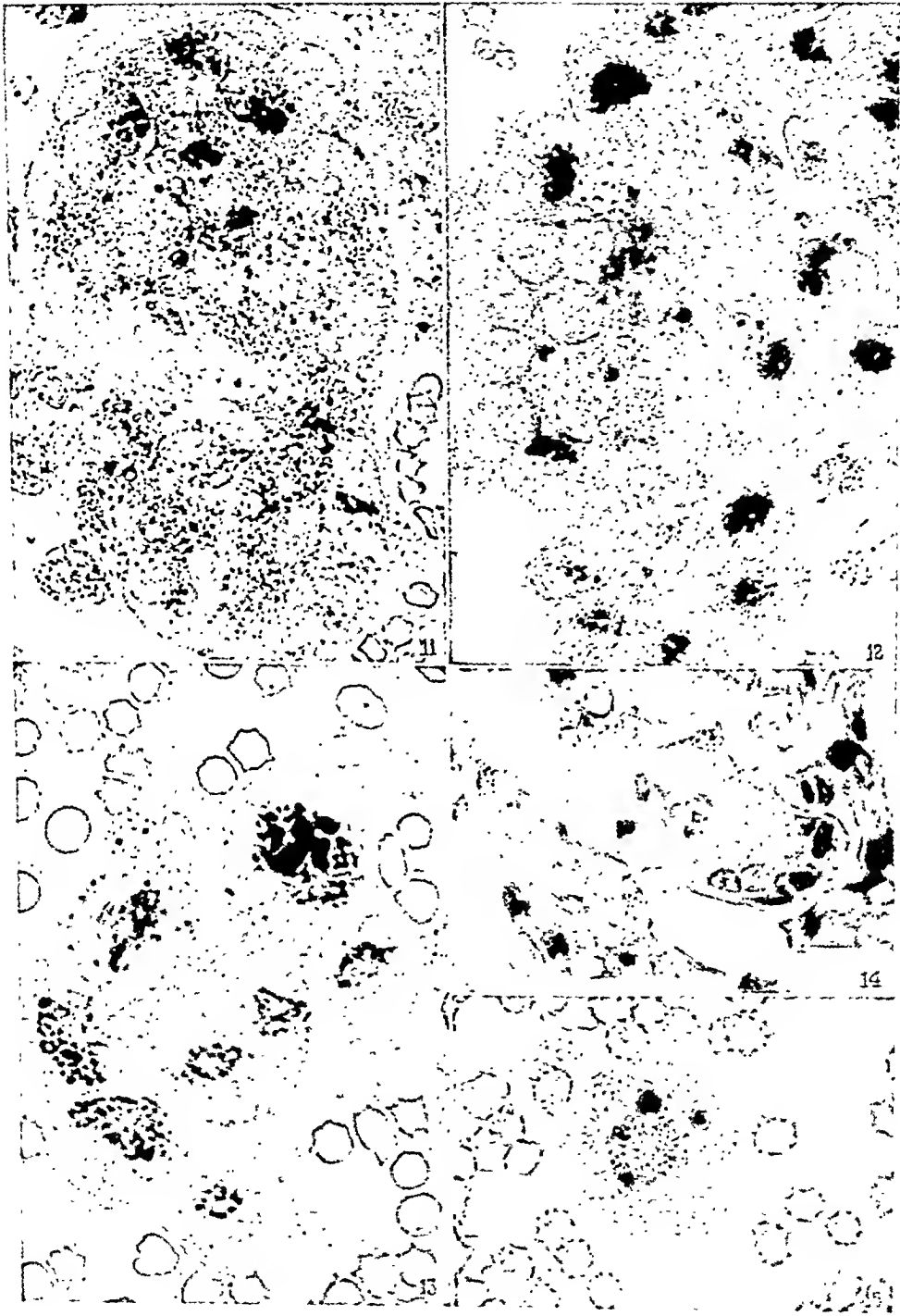
FIG. 12. Photograph of a foreign body giant cell in the blood with many monocytes and epithelioid cells about it. This animal, R 939, had received agar plus India ink which was injected under the skin and into the peritoneal cavity. Note the definite rosettes of neutral red bodies in the monocytes and epithelioid cells. Magnification $\times 750$. Stained with neutral red in supravital film.

FIG. 13. Photograph of an accumulation of monocytes about a foreign body in the blood of Rabbit R 967. The cell at the upper end of the figure is a clasmacyte. The cytoplasm of these cells could not be separated. They were apparently fused. A single monocyte may be seen nearby at the lower border of the figure. Magnification $\times 750$. Stained with neutral red by means of the supravital method.

FIG. 14. Photograph of a giant cell in a capillary of the lung of Rabbit R 579. Many giant cells could be demonstrated in the lungs of this and other animals similarly treated. Magnification $\times 750$. Stained in fixed section with hemotoxylin and eosin.

FIG. 15. Photograph of an epithelioid giant cell in the blood of Rabbit R 967. Note the unified central rosette and peripheral nuclei. Contrast with Fig. 11. Magnification $\times 750$. Stained with neutral red.





(Eosinophilic Multinucleated giant cells) (x 10,000)

ON THE MECHANISM OF OPSONIN AND BACTERIOTROPIN ACTION

V. EXPERIMENTAL TEST OF A THEORY OF TROPIN ACTION

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In previous papers we have reported that sera which have promoted the phagocytosis of acid-fast bacteria have regularly produced certain alterations in the bacterial surfaces (1, 2, 3) in corresponding degree. Conversely sera which produced these changes in the bacterial surfaces promoted phagocytosis correspondingly, although certain exceptions to the latter rule were found in using sera which had been altered by heating or aging. Both surface and tropin effects were clearly due to the deposit of certain substances from the immune sera on the surfaces of the homologous bacterial antigen; heating or aging in some cases impaired the phagocytosis-promoting action of these substances without similarly impairing their capacity to combine with the bacteria. The correspondence between the effectiveness of fresh rabbit immune sera in producing surface changes and in promoting phagocytosis was found to hold with sera of various strengths, during the course of immunization, and during the course of tuberculous infection and reinfection.

It then became of interest to determine the distribution of the substances responsible for the surface and tropin effects in the several fractions of immune sera. This subject is treated in the present paper.

Methods

Fractionation of Sera

Fresh serum, without preservative, is diluted with an equal volume of 0.85 per cent NaCl solution. To one volume of serum, diluted, one volume of 65 per cent

saturated ammonium sulfate solution is added. The mixture is shaken thoroughly and allowed to stand at room temperature for 15 minutes. It is then filtered in the ice box using a rather thick, soft filter paper. To the clear filtrate enough ammonium sulfate is added to bring the concentration of ammonium sulfate to 50 percent. This mixture is shaken thoroughly and allowed to stand 15 minutes at room temperature and filtered in the ice box. To the clear filtrate again enough ammonium sulfate is added to obtain a saturated solution; this is shaken thoroughly and after standing 15 minutes at room temperature, filtered in the ice box. The first precipitation separates the "euglobulin" fraction; the second the "pseudoglobulin" fraction, and in the third the albumin is brought down. After the filtration of each fraction the dried filter paper is temporarily stored in large petri dishes in the ice box. The precipitates are dissolved and washed off from the filter paper, using liberal amounts of distilled water. The solutions are then dialyzed for 4 days in the ice box, using parchment thimbles, and large quantities of water which are frequently changed. When dialysis is completed the fractions are evaporated to the original volume, with the aid of an electric fan, and enough sodium chloride is added to make 0.85 per cent solutions. If the solutions are not completely clear, centrifugation may be resorted to. If it is desired to separate the hydrophobic pseudoglobulins from the hydrophilic portion, after dialysis of the pseudoglobulin fraction, the contents of the dialyzing bag are centrifugalized at once. The sediment of hydrophobic pseudoglobulin can thus be separated by decantation.

Testing of Surface and Phagocytosis-Promoting Effects

The methods used in testing the effects of the sera and serum fractions were those already described in an earlier paper (1).

An even suspension of each antigen* was made and adjusted to a standard turbidity. Serial dilutions of each serum and serum fraction to be used were made. 1 cc. of antigen suspension was mixed with each dilution of serum or serum fraction. All tubes stood at room temperature usually for 30 to 90 minutes and were then put in the ice box over night. In the morning *agglutination* or sedimentation was read without shaking up the tubes. All tubes were centrifuged until clear; the supernatants were decanted, two drops of 0.85 per cent sodium chloride solution were added to each tube and all tubes were shaken up in the *resuspension* test. Excess of saline solution was added to each tube and all were again centrifuged. The supernatant fluids were again decanted, and the sensitized sediments brought to as even a suspension as possible in their original volume of saline. These suspensions of washed sensitized antigen were routinely used for *cataphoresis* and *interface* reactions, and for the tests of phagocytosis of washed antigen.

* The term "antigen" is for convenience applied in this paper to the whole bacteria; it is of course understood that the bacterial cell usually contains more than one antigen.

Phagocytosis of washed antigen was tested by mixing 0.2 cc. of polymorphonuclear leucocyte suspension with 0.2 cc. of each washed, sensitized antigen suspension in a vial and rotating all the vials for 15 minutes on a Robertson agitator. Phagocytosis in the presence of serum or serum-fraction dilution was conducted by rotating in each vial a mixture of 0.2 cc. of leucocytes suspension, 0.1 cc. of serum dilution and 0.1 cc. of stock antigen suspension (of twice the concentration of the washed suspension mentioned above). The making of smears, staining and counting in the phagocytosis tests were as already described.

Certain minor departures from the routine outlined were made from time to time, but these were unimportant. For instance resuspension and cataphoresis were occasionally tested with the particles suspended both in the original serum or serum fraction dilutions and also after washing.

Increased cohesiveness of the antigen surface due to sensitization is indicated by positive resuspension and interface reactions. Decreased surface electric potential difference is indicated by decreased velocity of migration in cataphoresis. Altered wetting properties of the antigen surface are indicated by the interface reaction. The degree of phagocytosis-promoting or tropin effect is indicated by comparison of the percentage phagocytosis of sensitized and control bacterial suspensions.

Correlation between Bacterial Surface and Tropin Effects Produced by Immune Sera and Their Fractions

The immune sera and their fractions have proved to be effective in the same order in altering the surface and in causing agglutination and phagocytosis of their homologous bacteria. The order of effectiveness has been uniformly: whole serum > euglobulin > pseudoglobulin > albumin. In only one of eleven experiments was any deviation from this order detected; in this experiment euglobulin was more effective than pseudoglobulin in some reactions, less effective in others.

As heretofore the correspondence between the surface changes and the agglutination and phagocytosis brought about by whole immune sera has been found in this work to be exceedingly close. The correspondence between surface, agglutinating and bacteriotropic effects has been exceedingly good also when the euglobulin or pseudoglobulin fractions of immune sera have been used. Of course the parallelism between surface and tropin effects was not perfect in all cases; for

instance the phagocytosis titers tended to run a little higher than those of the surface reactions when Arloing, Prague or chelonci strains were used as antigens, a little lower when Bovine III was antigen; also minor quantitative discrepancies occurred from time to time. Such occasional slight discrepancies are, however, inevitable in an extensive

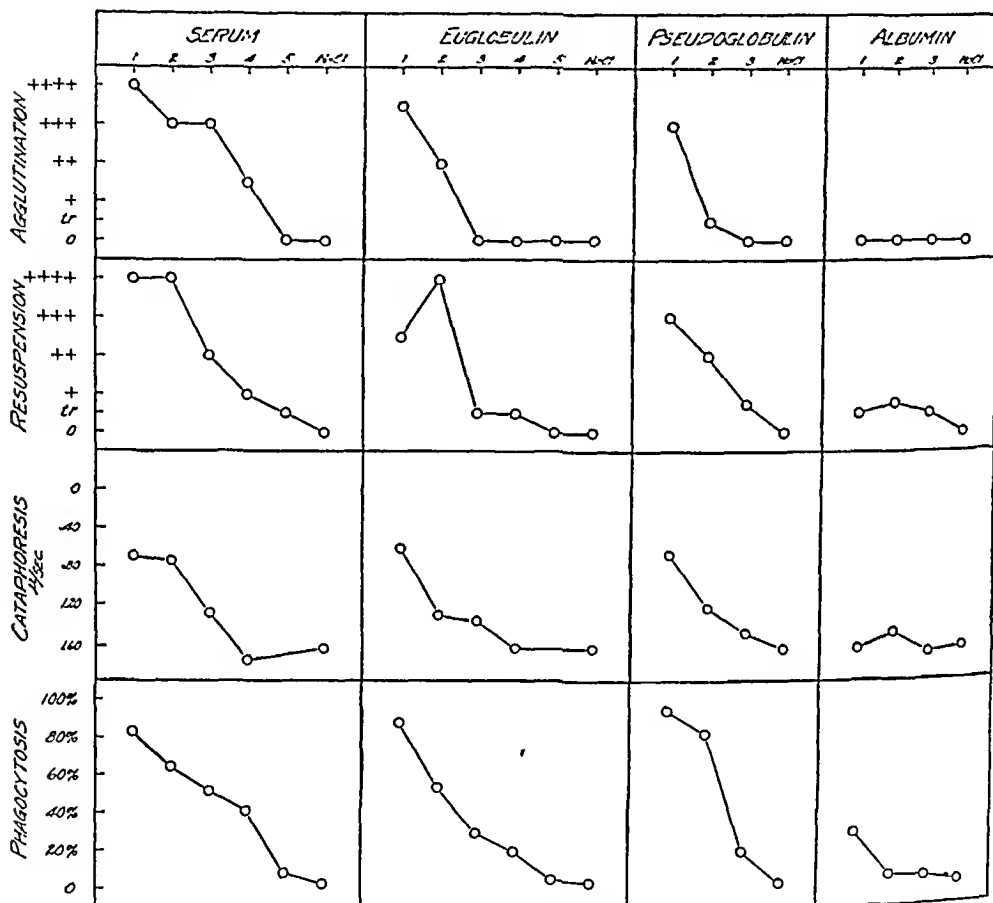


FIG. 1. Parallelism between surface changes and phagocytosis in presence of immune serum and its globulin fractions. Abscissae are successive dilutions of antiserum or serum fraction in powers of four. (Thus 3 is a dilution of $1:4^3$ or $1:64$.) *M. avium* (Arloing strain) and homologous antiserum.

experiment of this character in which the tests cannot be perfectly precise and necessarily include in each case variables not common to the other tests. With these slight reservations in mind the impressive fact has been the close correspondence between surface, agglutinating and tropin effects when whole serum or its euglobulin or pseudoglobulin fractions have been used.

In contrast to this were the results when the albumin fractions of antisera were used. The presence of serum albumin in the phagocytic mixture usually induced a moderate degree of phagocytosis. This effect was moderate at a dilution of albumin of 1 to 4, was weaker or

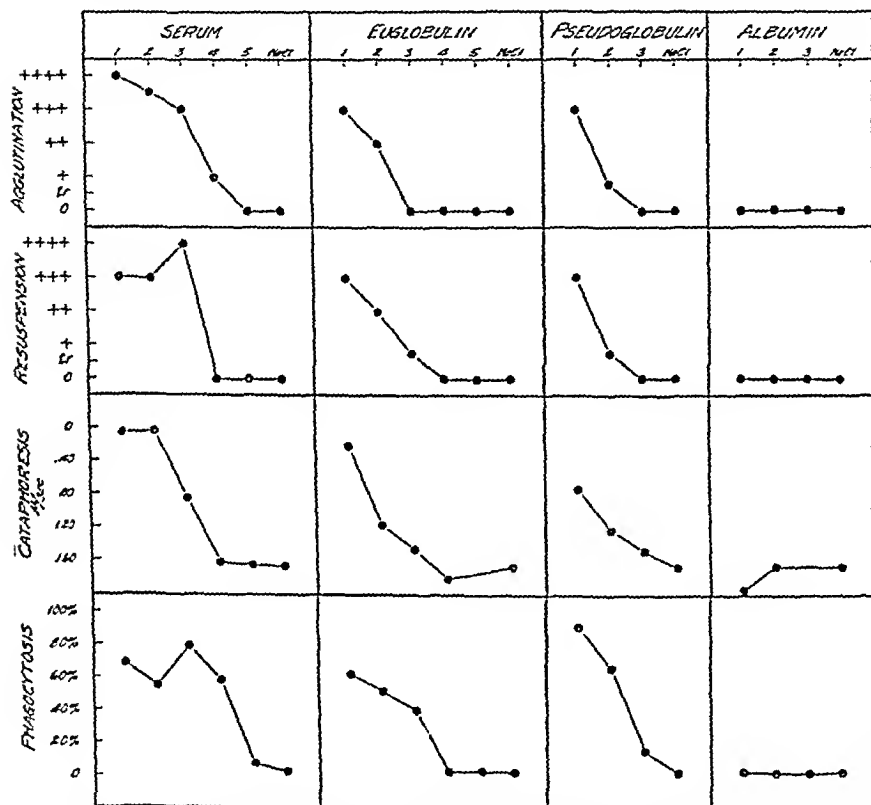


FIG. 2. Parallelism between surface changes and phagocytosis after sensitization with immune serum and its globulin fractions. The same antigen and antiserum as in Fig. 1. The antigen was sensitized and washed, then exposed to phagocytosis.

absent at dilutions of 1 to 16 and 1 to 64, and did not extend to higher dilutions. Serum albumin on the contrary caused only very slight and irregular surface or agglutinating effects and these were essentially out of correspondence with the phagocytosis-promoting effect.

TABLE I
The Effect of Washing upon the Phagocytosis of Bacteria Sensitized with the Globulin and Albumin Fractions of an Immune Serum

	Serum dilutions				NaCl contro
	1:4	1:16	1:64	1:256	
Series A: <i>M. chelonae</i> in anti-chelonae serum-euglobulin					
Agglutination.....	++++ to +++	++++ to +++	++++ to +++	++++ to +++	0
Resuspension.....	+++	+++	—	++	0
Interface.....	+++	+++ to ++	—	+	0
Cataphoresis, μ /sec.....	0	0	—	0.19	1.80
Phagocytosis, washed, %.....	90	97	—	92	4
Phagocytosis, unwashed, %.....	97	91	97	97	3
Series B: <i>M. chelonae</i> in anti-chelonae serum-pseudoglobulin					
Agglutination.....	++++ to +++	++++ to +++	+++ to +	0	0
Resuspension.....	++	+	+	0	0
Interface.....	++ to +	+ to tr.	\pm	—	—
Cataphoresis, μ /sec.....	0	0.61	1.11	1.53	1.98
Phagocytosis, washed, %.....	100	92	83	8	6
Phagocytosis, unwashed, %.....	98	87	91	16	3
Series C: <i>M. chelonae</i> in anti-chelonae serum-albumin					
Agglutination.....	0	0	0	—	—
Resuspension.....	tr.	tr.	0	—	—
Interface.....	0	—	—	—	—
Cataphoresis, μ /sec.....	1.61	1.65	1.41	—	—
Phagocytosis, washed, %.....	8	4	6	—	—
Phagocytosis, unwashed, %.....	54	7	3	—	—

Series D: *M. tuberculosis* in anti-chelonei serum-euglobulin

	++++ to ++	++	++	++	tr.
Agglutination.....	++++ to ++	++	++	++	0
Resuspension.....	++++ to ++	++	++	++	0
Interface.....	++++ to +++	++ to +	++	++	1.58
Cataphoresis, μ /sec.....	0	0	0	0	3
Phagocytosis, washed, %.....	27	30	11	10	45
Phagocytosis, unwashed, %.....	56	45	48	45	1

Series E: *M. tuberculosis* in anti-chelonei serum-pseudoglobulin

	++	++ to +	++ to +	++ to tr.	tr.
Agglutination.....	++	++ to +	++ to +	++ to tr.	+
Resuspension.....	tr.	++ to +	++ to +	++ to tr.	+
Interface.....	+	tr.	tr.	0.58	2
Cataphoresis, μ /sec.....	0	0.32	8	1.00	0
Phagocytosis, washed, %.....	38	15	25	14	0
Phagocytosis, unwashed, %.....	61	49	25	14	0

Series F: *M. tuberculosis* in anti-chelonei serum-albumin

	tr.	tr.	tr.	tr.	tr.
Agglutination.....	tr.	tr.	tr.	tr.	+
Resuspension.....	++ to +	tr.	tr.	tr.	+
Interface.....	0	—	—	—	+
Cataphoresis, μ /sec.....	0.85	1.14	1.11	1.11	+
Phagocytosis, washed, %.....	9	4	2	2	+
Phagocytosis, unwashed, %.....	28	11	10	10	+

The Effect of Washing the Sensitized Bacteria

Whether phagocytosis was conducted in the presence of the whole immune sera, or whether the bacteria were sensitized and washed and then exposed to the leucocytes, made very little difference. In an occasional experiment the percentage phagocytosis was slightly less after washing, but in the majority it was not affected. The same was true for the euglobulin and pseudoglobulin fractions. Illustrative experiments are given in Figs. 1 and 2 and Table I.

In contrast to the whole serum and globulin fractions, the phagocytosis-promoting effect of the serum albumin was in all cases much reduced or was abolished by washing. This constitutes a second point of differentiation between the phagocytosis-promoting effect of albumin and the true tropin effects of the globulin fractions. A third difference, that of specificity, is brought out in the paper following this.

The Effect of Heating the Sera before Fractionation

Each of three batches of immune serum was divided into two portions, of which one was heated at 56°C. for 30 minutes. The heated and unheated portions of each serum were fractionated and tested with the homologous bacterial strain.

The surface and tropin effects of an anti-avian (Arloing) and of an antichelonei serum were only very slightly if at all weakened by the heating except that, in the case of the antichelonei serum, a definite agglutination and phagocytic prozone appeared in the heated portion; there was no prozone in resuspension, interface or cataphoresis reactions. Such prozones in the other reactions have occasionally been noted in our work with immune sera heated to 56°C. for 30 minutes. They are evidently similar to the more pronounced agglutination prozones produced by Shibley (4) by heating antisera to higher temperatures. The phagocytosis-promoting properties of immune sera may then be impaired by heating, as well as by aging (2), even in instances in which the surface reactions indicate no impairment of the capacity of the serum to combine with antigen.

The surface and tropin effects of the euglobulin fractions of the immune sera were only very slightly if at all weakened by the heating. The surface and tropin effects of the pseudoglobulin fractions of the antisera were not appreciably weakened by the heating.

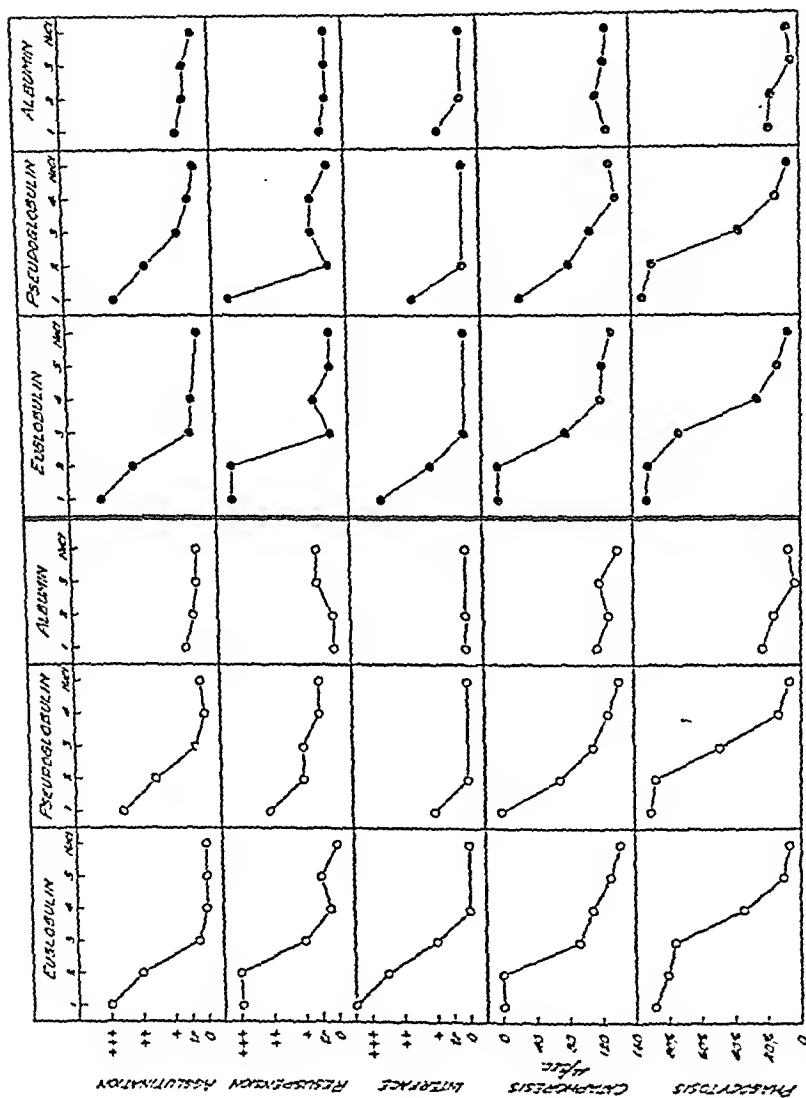


FIG. 3. Parallelism between surface changes and phagocytosis after sensitization with globulin fractions of immune serum. *M. arloing* (Arloing strain) sensitized with serum fractions and then washed. Open circles: fractions from Anti-Arloing serum heated for 30 minutes at 56°C. before fractionation. Black circles: fractions from same Anti-Arloing serum unheated.

DISCUSSION

It is clear, then, that the specifically active substances in our immune sera are distributed between the globulin fractions. The small phagocytosis-promoting effect of serum albumin differs in several respects from the tropin action of whole sera and their globulin fractions. The data lead very directly to the conclusion, therefore, that the interaction of antigen and fresh immune serum results in the deposit on the antigen surface of a substance or substances contained in the globulin fractions of the antiserum. The surface reactions, agglutination and increased phagocytosis are quantitatively correlated with this surface deposition, and without a doubt are consequences of the properties of the substances deposited on the antigen surface and of the special environing conditions of the several tests.

The question very naturally arises as to whether this conception of antibody action is in harmony with previous work; this is the case.

It was demonstrated by early investigations that antigens in sensitization acquire a deposit of material which at least has properties in common with the serum of the species from which the antiserum has come.

Ehrlich and Morgenroth (5) demonstrated in 1901 that immune sera produced by a given species, A, when injected into another species, B, led to the development of anti-immune sera which could neutralize the antibodies in immune sera in species A. Bordet (6) showed similarly that normal serum of species A injected into another species produced anti-sera which would neutralize the antibodies in sera of species A. Bordet further showed that such anti-immune sera could even interact with antibodies already united with their antigens and could alter the state of their union with antigen. Thus ox cells sensitized with rabbit immune serum and washed, were "cured" by treatment with anti-sensitizer produced by injection of normal rabbit serum into guinea pigs; addition of complement did not cause hemolysis of the sensitized ox cells. The interaction of sensitizer and anti-sensitizer was shown to be a true combination. Finally it was shown that normal rabbit serum could partially displace rabbit sensitizer from its union with anti-sensitizer.

Bacteria sensitized with immune horse-serum and injected into guinea pigs by Braun (7) made the guinea pigs anaphylactic to horse serum. Bacteria sensitized with immune horse-serum were stated to absorb the anaphylactic antibodies from the serum of guinea pigs and the precipitating antibodies from the sera of rabbits injected with horse serum. Altmann (8), by immunizing animals with sensitized red blood cells, thoroughly washed, obtained sera which gave precipitation and

complement fixation with serum of the species from which the sensitizer had come.

More recent studies by physical-chemical methods have closely associated the sensitizing substance or substances with the globulin of the immune serum.

Porges (9) reported that bacteria treated with a large excess of agglutinin were precipitated by salts in a range of concentration characteristic for serum globulin rather than for the unsensitized bacteria.

Coulter (10, 11) reported that strong sensitization with immune rabbit serum shifted the agglutination optimum of sheep's red blood cells from its value with normal cells of pH 4.75 to a pH of about 5.3; the latter value is close to the isoelectric point of serum globulin. The maximum combination of hemolytic sensitizer from rabbit serum with sheep red blood cells (and minimum dissociation) were stated to occur also at a pH of about 5.3.

The work of Northrop and De Kruif (12), and Northrop and Freund (13) showed that the agglutinating substances from immune sera form a deposit on the surface of the homologous bacteria.

Red blood cells (14), acid-fast bacteria (15) and certain spirochetes (16) have been shown by Mudd and Mudd to have surfaces whose wetting properties are suggestive of a high lipin content. Sensitization of each of these antigens with homologous immune serum gives it an altered surface whose wetting properties are like those of protein.

Shibley (17) found that a variety of bacteria whose surface electric potential differences vary widely, were all brought by sensitization to essentially the same potential difference. The electric behavior of strongly sensitized bacteria closely paralleled that of serum globulin.

Eagle (18) showed that the physical constants for adsorption by sensitized bacteria and red blood cells were closely similar to those for adsorption by immune precipitate. The work of Moll (19), Welsh and Chapman (20), Ottensooser (21), Wu and his associates (22), and Marrack and Smith (23) indicate that a large part of immune precipitate is protein material from the globulin fraction of the anti-serum.

Heinemann and Gatewood (24) demonstrated that the bacteriotropins in anti-streptococcic and antigonococcic horse sera were precipitated with the globulins.

The work cited thus shows by a number of independent methods that an antigen in specific sensitization receives a surface deposit of a substance or substances which at least have several points of resemblance to serum globulin.

The contributions of Rhumbler (25), Tait (26) and especially those

of Fenn (27) and of Ponder (28) have shown on theoretical grounds that a major requirement for phagocytosis is that the particles to be ingested shall have a surface upon which the leucocytes will spread.

We conclude, therefore, both from our own work and from that of earlier investigators, that *the contact of antigen with fresh homologous immune serum results in the deposit on the antigen surface of a substance or substances contained in the globulin fractions of the anti-serum; as a consequence of this surface deposit leucocytes can spread upon and engulf the antigen.*

The conception of antibody action to which we and other students of the physical-chemistry of immune reactions have been led, represents a synthesis of certain elements from the views of the older schools associated, respectively, with the names of Ehrlich and of Bordet. Practically all investigators are agreed, so far as we know, that the combination of antigen and antibody is determined by specific chemical affinities. The beautiful demonstration that the specificity of antigen and antibody may be determined by the spacial configurations about a single carbon atom securely establishes this point of view (29, 30). On the other hand we find no evidence to support the conception of special reacting groups on the antibody responsible for bringing about the several immune reactions. The evidence, at least in the systems with which we have worked, supports the conception of precipitation, agglutination, phagocytosis, and the changes detected in the interface and cataphoresis reactions as consequences, under the several conditions of test, of the properties of the same substances deposited on the antigen surface. The combination of antigen and antibody then is specific; the consequences of that combination are non-specific expressions of certain physical-chemical attributes of the immune substances, and the phenomena exhibited are various rather because of various environing conditions than because of differences in the attributes of the immune substances. Further evidence in support of this conclusion will be presented in the paper following.

We do not wish at the present time to extend this discussion to include lytic or antitoxic reactions. Even in such cases it seems not improbable, however, that the phenomena exhibited may depend more upon the properties of the antigens than upon any special peculiarities of the specific antibodies involved.

SUMMARY

Antisera against several strains of acid-fast bacteria have been separated into their euglobulin, pseudoglobulin and albumin fractions. The globulin fractions have been found to possess the essential properties of bacteriotropic sera: thus they alter the bacterial surface properties, and, in quantitative correspondence, cause agglutination and phagocytosis; these several effects withstand washing of the sensitized bacteria; the effects are little if at all affected by inactivation of the antisera before fractionation; the combination of antibody and antigen is serologically specific.

The conclusion is drawn that the contact of antigen with fresh homologous immune serum results in the deposit on the antigen surface of a substance or substances contained in the globulin fractions of the antiserum; as a consequence of this surface deposit leucocytes can spread upon and engulf the antigen.

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ON THE MECHANISM OF OPSONIN AND BACTERIOTROPIN ACTION

VI. AGGLUTINATION AND TROPIN ACTION BY PRECIPITIN SERA. CHARACTERIZATION OF THE SENSITIZED SURFACE

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In the preceding communication we have advanced a theory of tropin action and offered in its support evidence that we feel to be convincing. The effectiveness of immune sera in agglutinating, altering the surface properties and promoting the phagocytosis of antigens has been found to be dependent upon the presence of substances in the globulin fractions of the serum which combine with and form a deposit on the surface of the antigen. Phagocytosis, the characteristic behavior of the sensitized antigens in resuspension, interface and cataphoresis tests, and agglutination follow essentially as consequences of the properties of the substances deposited on the antigen surface.

In the present communication we shall describe building up artificial surfaces and obtaining agglutination, characteristic surface properties and phagocytosis in accordance with prediction from the theory. In this phase we are following and extending the work of F. S. Jones (1, 2). We find with Jones that precipitin sera will cause the agglutination and phagocytosis of collodion particles previously treated with homologous precipitinogen. The active substances are found both in the euglobulin and pseudoglobulin fractions of the antisera. Precipitation, agglutination, altered cataphoretic properties and phagocytosis are brought about in approximately corresponding degree. And finally the properties of maximally sensitized precipitinogen-treated collodion particles, of specific immune precipitate, and of maximally sensitized acid-fast bacteria are, within the limits of our methods, identical.

Thus we have powerful evidence in support of a rational conception of these diverse serological phenomena as all special consequences of one underlying phenomenon. The combination of antigen and antibody is determined by specific chemical affinities. The effects following this combination, at least with the antigens we have thus far studied, namely precipitation, agglutination, changes in surface properties and phagocytosis are consequences primarily of the properties of the antibody-protein combined with and deposited upon the antigen surface. The actual phenomena exhibited as a consequence of the deposit of the antibody-protein on the antigen surface of course depend also upon accessory factors such as electrolytes, temperature, the presence of leucocytes and other environing conditions.

Experimental Methods

Immune Sera.—Rabbits were injected five times usually at 5-day intervals with solutions of the antigen. These were crystalline egg albumin,* edestin,* casein (Hammarsten), human serum and horse serum. The egg albumin and human serum and their antisera were most satisfactory.

Preparation of Precipitinogen-Treated Collodion.—Loeb (3) showed that protein could be adsorbed on the surface of collodion particles. Brilliant application of this fact to serology was made by F. S. Jones (1, 2).** We have followed essentially the technique of Jones. Spherical collodion particles of about 1 to 2 μ in diameter were furnished us through the kindness of Dr. M. Kunitz. One volume of a dense suspension of these particles was allowed to stand usually for several hours in contact with one volume of human or horse serum or of a saturated solution of the protein. The collodion-precipitinogen mixture, after sufficient contact, was centrifugalized, the supernatant fluid decanted and the sediment resuspended in excess of saline. The suspension was again centrifugalized, the supernatant fluid decanted and the sediment resuspended in 0.85 per cent saline.

Tests.—The precipitinogen-treated, washed collodion particles were set up with serial dilutions of the precipitin sera or of the globulin or albumin fractions of these sera. Agglutination was read at short intervals and often again after keeping the tubes overnight in the ice box. Resuspension was often omitted, but was otherwise done according to the usual technique (5). The interface reaction could not be done since the collodion particles themselves are not wetted by oil.

* For the egg albumin we are indebted to Dr. J. Freund, and for the edestin to Professor D. Wright Wilson.

** Specific agglutination of protein-treated collodion particles was demonstrated independently by Freund (4).

Cataphoresis was carried out as previously described (5).^{*} In the earlier experiments the test particles were suspended in 0.85 per cent sodium chloride solution. In later work the particles were suspended in $M/50$ acetate buffer of pH 5.2. When isoelectric point determinations were made the particles were suspended in two or more buffers whose reactions were graduated in intervals of 0.4 pH. Buffers were found such that the test particles moved toward the anode in one, toward the cathode in the next in series. The isoelectric point was then estimated by interpolation.

The phagocytosis tests, staining and counting were as previously described (5). The use of essentially the same methods of detecting phagocytosis of collodion particles as had been used with acid-fast bacteria was made possible by the earlier observation of Freund (6) that collodion particles are acid-fast by the ordinary Ziehl-Neelsen technique.

Precipitin tests were set up by adding 0.2 cc. portions of the precipitin serum or serum fraction to 1 cc. each of the serial dilutions of the precipitinogen solution.

Fractionation of the Precipitin Sera.—Fractionation was carried out as previously described (7).

Agglutinin and Tropin Action by Precipitin Sera; Further Verification of the Theory of Tropin Action

Precipitation tests with the dissolved proteins, and agglutination, cataphoresis and phagocytosis tests with the proteins adsorbed on collodion particles were performed as routine with all of the antiserum fractions and often also with the whole antisera. In the majority of experiments two antigens and their antisera were included and both homologous and heterologous combinations were tested. The data were finally analyzed with reference to certain questions designed, like those in the preceding paper, to test deductions from our theory of tropin action. In addition to verifying the theory the data have yielded strong support of the unitarian hypothesis of antibody action (8).

^{*} A Zeiss water-immersion lens of 0.75 n.a. and 1.9 mm. working distance has been found to be in certain respects superior to the objective previously described. The water-immersion feature is a nuisance; however, for studying particles which settle out quickly, this may be more than compensated for by the advantages of more critical focus and the greater magnification afforded by the water-immersion lens.

The questions and the results of analyses of the data are given below. Details have for the most part been omitted.

I. When precipitinogen-treated collodion particles are sensitized with immune serum or one of its fractions, what is the order of relative effectiveness in (1) phagocytosis, (2) agglutination, (3) cataphoresis, (4) precipitation?

Whole serum was found generally to be more effective in bringing about all of the reactions than either of its globulin fractions; both globulin fractions were strikingly more effective in all of the reactions than the albumin fraction. In precipitation and phagocytosis the euglobulin and pseudoglobulin fractions were about equally effective; in agglutination pseudoglobulin was oftener more effective than euglobulin, in cataphoresis euglobulin was oftener more effective than pseudoglobulin. With the exception of this slight discrepancy in the relation of the euglobulin and pseudoglobulin fractions, therefore, the sera and fractions were effective in the same order in all of the reactions.

It has been supposed that the euglobulin fraction contains all the precipitin (9); certainly in our work we have found it fairly equally distributed between the euglobulin and pseudoglobulin fractions. Possibly it is not irrelevant to the perennial controversy over the distribution of antibodies in the globulin fractions to point out that two of the chief students of globulin chemistry, Chick (10) and Sørensen (11), believe that a clean-cut separation of euglobulin and pseudoglobulin is impracticable. According to Sørensen, "neither fractionation nor dialysis nor a combination of the two methods results in a complete separation of the two globulins." He believes that the euglobulin and pseudoglobulin are associated as a labile compound E_pP_q .

II. When precipitinogen-treated collodion particles are sensitized with immune serum or one of its fractions, are the effects in promoting phagocytosis approximately proportional to the effects on 1) agglutination, 2) cataphoresis, and 3) precipitation?

The correspondence between tropin, agglutinating, cataphoretic and precipitating effects with whole serum and with both globulin fractions was excellent in practically all experiments. There was not good correspondence between the several tests when the serum albumin fraction was used. The results are thus precisely similar to those obtained with acid-fast bacteria (7).

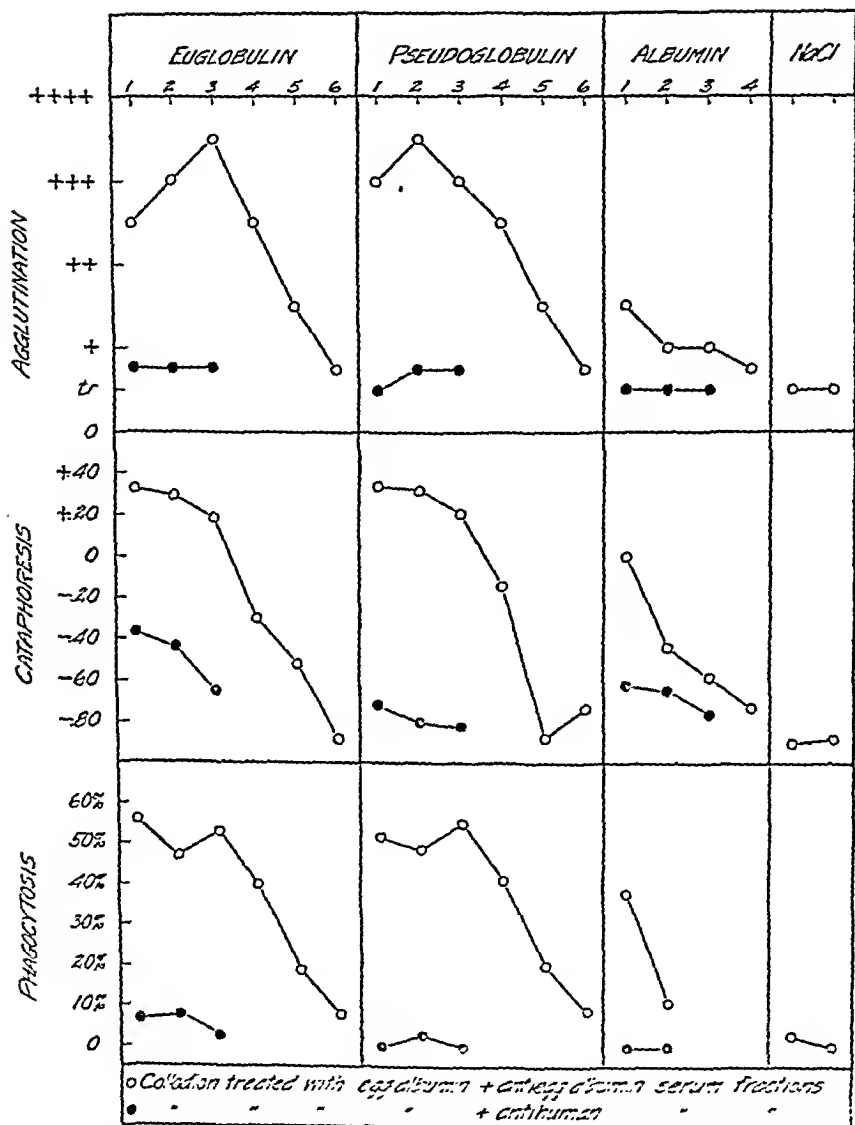


FIG. 1. Parallelism between agglutination, cataphoresis and phagocytosis of precipitinogen-treated colloidion particles after sensitization with globulin fractions of precipitin sera. Abscissae are successive dilutions of antiserum or antiserum fraction in powers of four. (Thus 4 is a dilution of 1:4⁴ or 1:256.) All particles were washed after sensitization. Cataphoresis was conducted in 1/50 acetate buffer of pH 5.2; graphed as μ /sec. per volt/cm. Note specificity of the reactions.

cal sense by Hardy (12) as the reaction at which no migration of the test substance occurs in an electric field. The isoelectric point for the case of a pure amphoteric substance later received mathematical definition in terms of the acid and basic dissociation constants of the substance (13). We shall, however, use the term "isoelectric point" in the original sense of Hardy.

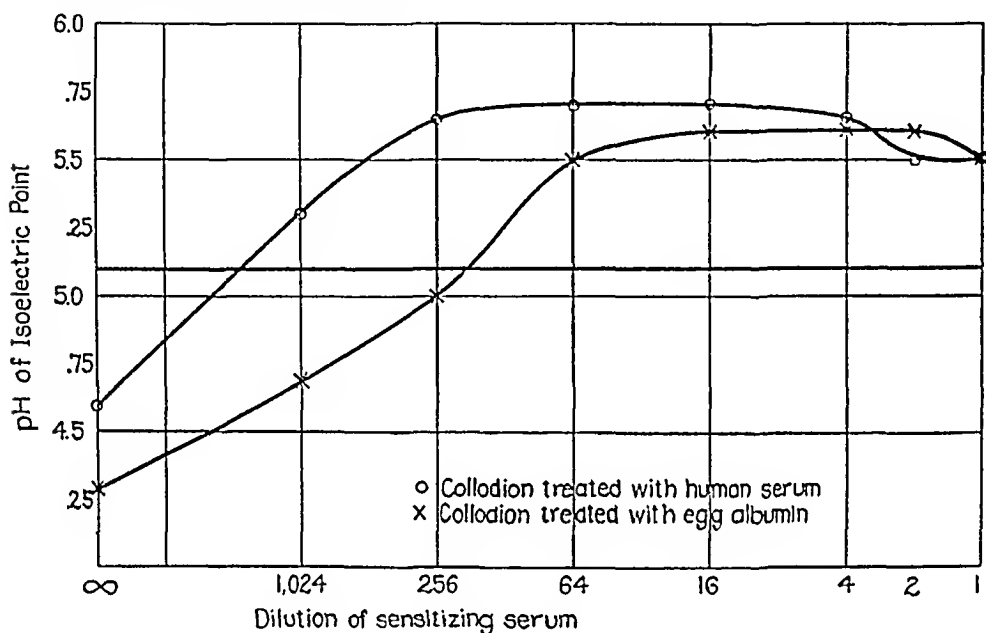


FIG. 3. Progressive change of the isoelectric points of precipitinogen-treated collodion particles with sensitization by homologous precipitin sera. The isoelectric points of the protein-treated collodion particles are about pH 4.3 and 4.6 respectively. After exposure to homologous immune serum in progressively increasing concentrations, the isoelectric points shift to plateau values of pH 5.6 and 5.7. There is a slight prozone with the highest serum concentrations. The horizontal line at pH 5.1 is the mean value for eleven determinations of the isoelectric point of serum euglobulin.

Collodion particles show a strong negative charge in the electric field. When treated with protein by the technique we have followed the particles are isoelectric at a reaction somewhat on the acid side of the isoelectric point of the protein used. This indicates that protein has been adsorbed on the surface of the collodion. The fact that the isoelectric point is on the acid side of that of the protein indicates that the charge is a resultant of that associated with the collodion and that

due to the protein. This supports the conclusion reached by Jones (2) on other grounds that the protein under such circumstances does not form a complete film on the particle.

When such protein-treated collodion particles are sensitized by strong precipitin sera homologous with the protein used, the isoelectric point is shifted to the alkaline side. The shift is progressively greater with progressing concentrations of sensitizing serum until a plateau value of

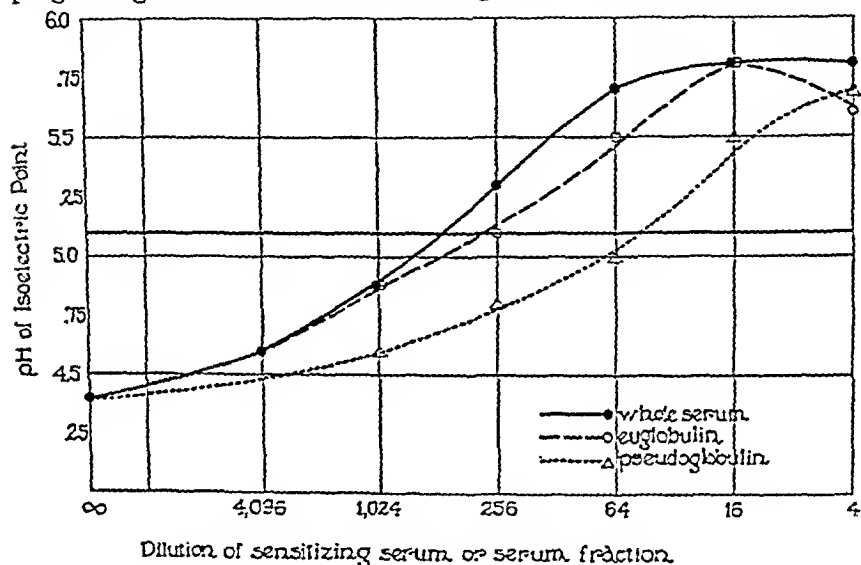


FIG. 4. Progressive change of the isoelectric points of human-serum-treated collodion particles with sensitization by homologous precipitin serum and its globulin fractions. The globulin fractions as well as the whole serum bring the isoelectric point of the antigen within the zone of pH 5.5 to 5.8. Figs. 1, 2, 4 and 5 and Table I all represent data from the same experiment.

pH 5.6 to 5.8 is reached. This effect is seen in Fig. 3. With sera of lower titer greater concentrations of sensitizing serum are required to bring the isoelectric point of the sensitized particle to the plateau value, as seen in Figs. 4 and 5. These figures show that a similar effect is produced by the euglobulin and pseudoglobulin fractions of the immune sera, but that greater concentrations are required to bring it about than with the whole serum. With still weaker immune sera the maximum value reached by the isoelectric point may be below pH 5.6.

The isoelectric points of acid-fast bacteria have been shown in another place (14) to undergo a similar change with serum sensitization. The isoelectric points of the unsensitized bacteria studied were exceedingly low, below pH 2.5; with sensitization by increasing strengths of homologous antiserum the values of the isoelectric points shifted to the alkaline side until values of pH 5.5 to 5.8 were reached.

The isoelectric points of specific precipitates obtained by the interaction of the dissolved proteins and their homologous precipitin sera have

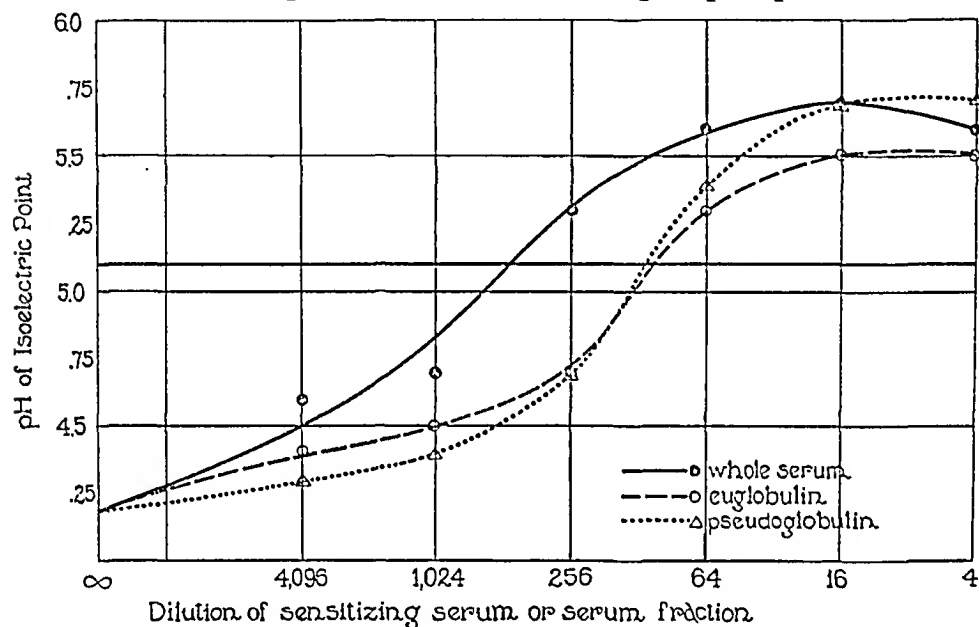


FIG. 5. Progressive change of the isoelectric points of egg-albumin-treated collodion particles with sensitization by homologous precipitin serum and its globulin fractions. The globulin fractions as well as the whole serum bring the isoelectric point of the antigen within the zone of pH 5.5 to 5.8.

also been studied. The results are given in Tables I, II and III. It is evident that the precipitates obtained in the zone of approximately optimal proportions of antigen and antibody are isoelectric in the same range of hydrogen ion concentrations as are maximally sensitized acid-fast bacteria or precipitinogen-treated collodion particles, namely pH 5.5 to 5.8.

With excess of either antigen or antibody the isoelectric points of the immune precipitates have tended to be at slightly lower pH

values.* An analogous instance in which sensitization with the highest concentrations of a high titer immune serum has resulted in a reduction of the isoelectric point to slightly below the maximum value

TABLE I

Isoelectric Points of Specific Precipitates

Immune serum	Dilution of antigen	Precipitate, amount	Isoelectric point of precipitate, unwashed, pH	Isoelectric point of precipitate, washed, pH
Antiegg albumin.....	1:10	+++ to ++	5.15	5.5
" "	1:100	+++	5.35	5.55
" "	1:1000	+++	5.4	5.75
" "	1:10,000	+	—	—
" "	1:100,000	tr.	—	—
Euglobulin from the above serum		—	5.0	5.0
Antihuman serum.....	1:10	+++ to ++	5.4	5.55
" "	1:100	++++ to +++	5.6	5.75
" "	1:1000	++++ to +++	5.4	5.7
" "	1:10,000	++ to +	—	—
" "	1:100,000	+	—	—
Euglobulin from the above serum		—	4.8	4.95

1 cc. each of progressive dilutions of egg albumin solution and of human serum were mixed with 0.2 cc. each of their respective homologous precipitin antisera. Euglobulin was obtained from the same precipitin sera by dilution and acidification. The precipitates were first concentrated by centrifugalization (unwashed); they were then suspended in distilled water, centrifugalized, the supernatant decanted and the sediment resuspended in distilled water (washed); a drop or two of the suspensions were added to several cubic centimeters of dilute acetate or phosphate buffer for the isoelectric point determinations.

is given in Fig. 3. Similar instances have occurred a sufficient number of times in as yet unpublished experiments with potent antisera so

* Incidentally these results show that the often-cited analogy between the zone phenomena in specific precipitation and that seen in the reciprocal precipitation of an electronegative and electropositive colloid is both superficial and misleading. The antigen, the precipitate, and, as far as present evidence shows, the antibody are all electronegative at the hydrogen ion concentration of blood serum.

TABLE II
Isoelectric Points of Specific Precipitates

Antiserum	Dilution of antigen	Precipitate, amount	Isoelectric point of precipitate, pH
Whole antihuman serum.....	1:10	++	5.4
“ “ “	1:100	++++	5.7
“ “ “	1:1000	+++	5.4
“ “ “	1:10,000	++ to +	—
“ “ “	1:100,000	tr.	—
Antihuman serum euglobulin.....	1:10	++	—
“ “ “	1:100	+++	5.3
“ “ “	1:1000	+++	5.5
“ “ “	1:10,000	++	—
“ “ “	Euglobulin control	+	—
Euglobulin obtained from the above serum by dilution and acidification		—	5.0

1 cc. each of progressive dilutions of human serum were mixed with 0.2 cc. each of whole antihuman precipitin serum or of the euglobulin fraction of this precipitin serum. Euglobulin was obtained from the same precipitin serum by dilution and acidification. The precipitates were washed and isoelectric points were determined as described under Table I.

TABLE III
Isoelectric Points of Specific Precipitates

Dilution of antigen	Precipitate, amount	Isoelectric point of precipitate, pH
1:10	+++	5.4
1:100	++++	5.65
1:1000	+++	5.7
1:10,000	+	5.45
—	Euglobulin	5.25

2 cc. each of progressive dilutions of an egg albumin solution were mixed with 0.4 cc. each of anti-egg-albumin precipitin serum. Euglobulin was obtained from the same precipitin serum by dilution and acidification. The immune precipitate and the euglobulin were washed in distilled water; their isoelectric points were determined in dilute acetate buffers.

that it seems unlikely that they can be dismissed as accidental. Heidelberger and Kendall (15) have recently successfully treated the precipitin reaction between *Pneumococcus* III specific carbohydrate and its corresponding antibody in terms of simple mass action equations. It seems probable that in our experiments the rise of the isoelectric points through a maximum with increasing relative concentrations of antibody may correspond to the transition from the compounds AS_3 , through AS_2 to AS as described by Heidelberger and Kendall. Study should be directed explicitly to this point.

TABLE IV

Acid-fast bacteria + homologous antisera or their globulin fractions, result in	{ altered surface* agglutination phagocytosis
Protein-treated collodion + homologous antisera or their globulin fractions, result in	{ altered surface* agglutination phagocytosis
Protein solutions + homologous antisera or their globulin fractions, result in	{ immune precipitate*
Sheep erythrocytes + homologous antisera, result in	{ altered surface* agglutination phagocytosis

* Properties of altered surface and of immune precipitate:

1. Cohesion high.
2. Wetting properties characteristic of protein.
3. Isoelectric point at pH 5.5 to 5.8.

The wetting properties of immune precipitate when examined in an oil-water interface are also similar to those of maximally sensitized acid-fast bacteria. These properties are characteristic* for protein; for instance globulin, egg-albumin, casein or edestin particles examined in such interfaces exhibit wetting properties similar to those of the immune precipitate or the maximally sensitized bacterial surface.

The analogy between immune precipitate and the maximally sensi-

* Although characteristic, no claim is made that these wetting properties are specific for protein.

tized surface of particulate antigen is rendered virtually complete by the evidence brought forward by Cromwell and Centeno (16) to indicate that specific precipitate is phagocytized.

The chief results of the interaction of antigen and antibody in the systems we have studied are summarized in Table IV.

Earlier work on the isoelectric points of sensitized antigens is reviewed elsewhere (14). The work which has hitherto advanced farthest in characterizing the properties of the sensitized surface, that of Shibley (17), seemed to show that the surface was coated with a film of denatured serum globulin. That the sensitized surface has several points

TABLE V
Isoelectric Points of Eoglobulin Samples

Source	Method of preparation	Isoelectric point, pH
Antiegg-albumin Rabbit 29-19	Ammonium sulfate fractionation	5.2
Anticasein Rabbits 32-97 and 33-72	" " "	5.1
Antiegg-albumin Rabbit 29-19	Dilution and acidification	5.0
Antihuman serum Rabbit 31-77	" " "	4.9
Antihuman serum Rabbit 31-77	" " "	5.0
Antiedestin Rabbits 32-96 and 32-98	" " "	5.05
Antiegg-albumin Rabbit 29-19	" " "	5.2
Antiedestin Rabbit 32-96	" " "	5.2
Antiedestin Rabbit 32-98	" " "	5.2
Antiegg-albumin Rabbit 29-19	" " "	5.1
Antihuman serum Rabbit 31-77	" " "	5.1
Average		5.1

of resemblance to a film of serum globulin has been brought out also by the studies of Coulter (18), of Mudd and Mudd (19) and of Eagle (20). Moreover the sensitizing substance or substances are well known to be associated with the globulin in the fractionation of the serum. However, the familiar fact of possessing specific affinities for homologous antigen constitutes one obvious point of distinction between the antibody and the ordinary serum globulin. A further distinction between the properties of the sensitized surface and the serum globulin is brought out in Tables I to V and Figs. 3 to 5.

In Table V are given the isoelectric points of eleven samples of

euglobulin obtained from the precipitin sera. These were usually precipitated by diluting a small volume of the serum with about fifteen volumes of distilled water and adding a few drops of one-tenth normal hydrochloric acid. Two samples were obtained by ammonium sulfate fractionation. The values of the isoelectric points are seen to be in all cases within a range from pH 4.9 to 5.2, with an average of 5.1. The average value is plotted as a horizontal line at pH 5.1 in Figs. 3 to 5. It is thus evident that the isoelectric points of maximally sensitized acid-fast bacteria and precipitinogen-coated collodion particles and also of immune precipitate lie well to the alkaline side of the range of values for the isoelectric points of euglobulin samples precipitated as described from the same antisera.

A greater difference in the same direction has been reported by Felton (21) between the minimum solubilities of antibody-protein and the non-specific globulin of antipneumococcus horse sera. The minimum solubilities of Felton's antibodies lie between pH 6.6 and 6.8. Several interpretations are possible for the isoelectric values of 5.5 to 5.8 we have found for the maximally sensitized surface and for the specific precipitate. Conceivably these values might represent the values of the isoelectric points of the antibodies of rabbit serum; or these values might be a resultant of the isoelectric points of a more alkaline protein analogous to Felton's with more or less non-specific serum globulin adsorbed upon it; or the uncombined antibody might be isoelectric at a higher or lower pH than 5.5 to 5.8 and obtain these values because of changes involved in the union with antigen. Answers to these questions will have to await further investigation.

Useful conceptions of the nature of antibodies have been proposed by Locke, Main and Hirsch (22) and by Manwaring (23). The denaturation of the antibody-protein when deposited upon the antigen surface has been discussed in references 17, 19 and 24.

Throughout this work we have had capable technical assistance from Mr. H. J. Henderson.

SUMMARY

As a further test of the theory of tropin action proposed in the preceding paper artificial surfaces have been prepared, and have been found to be phagocytized according to prediction from the theory.

Protein was adsorbed on collodion particles according to the technique of F. S. Jones. These particles were then agglutinated and prepared for phagocytosis by the corresponding protein precipitin sera. The precipitating, agglutinating, surface and tropin effects for each serum or serum globulin fraction have been found to be in satisfactory quantitative correspondence. All of these effects were serologically specific; all remained almost unaffected by inactivation of the immune sera for 30 minutes at 56°C. or by washing of the particles after sensitization.

The surfaces of particles maximally sensitized by homologous rabbit immune serum or one of its globulin fractions have shown certain characteristic properties, *i.e.*, they were cohesive, had wetting properties characteristic for protein, and were isoelectric at pH 5.5 to 5.8. The same set of properties were found for immune precipitate in the zone of maximal precipitation. The same properties have also been found for maximally sensitized acid-fast bacteria, and for maximally sensitized sheep erythrocytes.

These results indicate, we believe, that precipitation, agglutination, the surface changes and increased phagocytosis are all consequences of one underlying phenomenon. This phenomenon is the specific chemical combination with, and deposit on the surface of the antigen of antibody protein. The several serological reactions then follow as consequences of the properties of the sensitized surface and of the special environing conditions.

The antibody is contained in the globulin fractions of immune serum, and appears to be a globulin with physico-chemical differences from normal serum globulin.

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THE BACTERIOSTATIC ACTION OF CERTAIN COMPONENTS OF COMMERCIAL PEPTONES AS AFFECTED BY CONDITIONS OF OXIDATION AND REDUCTION

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When *Pneumococcus* is seeded into plain broth, it is necessary to use a fairly large inoculum in order to insure the initiation of growth. The size of this inoculum increases with the amount of medium to be seeded; as a rule, 0.01 to 0.1 cc. of a plain broth culture is the minimum seeding to be used for 5 cc. of medium. These inocula contain at least 1,000,000 to 10,000,000 cells. What are the factors which make such a large number of cells necessary? Under what conditions would one single cell grow?

An answer to these questions will probably result in a better understanding of the physiology of the pneumococcus cell. But in addition, the preparation of an "ideal" medium (and no medium is "ideal" which does not allow the growth of a single cell) would be of great value in the study *in vitro* of the bacteriostatic and bactericidal properties of the body fluids of immunized and naturally resistant animals. However, the present work was undertaken to meet a purely practical need. During the past few years, considerable difficulties have often been encountered in the preparation of media suitable for the growth of *Pneumococcus*. Many lots of broth were found to be unable to support growth even when large inocula were used. Yet it has been observed that when various lots of media are prepared from the same meat infusion, but with the addition of different brands of peptones, these various lots differ in their ability to support growth of *Pneumococcus*, depending upon the kind of peptone employed; in all cases, growth would occur, even with very small inocula, provided a sufficient amount of neutral cysteine or thioglycollic acid was added. It seems therefore that the inability to support growth depends upon

some bacteriostatic property of the peptone and that this effect may be overcome by the addition of reduced thioacids.

It is known in a general way that the living cell can greatly affect the conditions of its environment. In particular, it may be recalled that pneumococcus cells acting on certain metabolites bring about the formation of actively reducing substances (1, 2). If, however, *very small inocula* be employed, the influence of the reducing action of the bacteria can be considered negligible. Advantage may be taken of this observation in devising an experimental method for the study of those properties of the medium which affect the initiation of growth.

Experimental Methods

Cultures.—All experiments were carried out with a strain of Type II Pneumococcus (D/39/79) and an R culture derived from Type II (D/39/R). Occasionally, control experiments were carried out with strains of Type I and Type III Pneumococcus. The results being always substantially the same, only the data obtained with the Type II strain are presented in this article.

Unless otherwise stated, the inoculum consisted of 0.0001 cc. of a 12-hour culture in plain broth.

Ingredients Used in the Preparation of Media.—The beef infusion was prepared as usual, enriched with 0.03 per cent glucose, adjusted to pH 7.8 and autoclaved.

“Tryptophane broth” is the commercial name of a dehydrated preparation of casein hydrolysed with acid. Solutions of 1 per cent of this dehydrated medium were enriched with 0.03 per cent glucose, adjusted to pH 7.8, and autoclaved.

Peptone solutions employed were of 10 per cent concentration; they were adjusted to pH 7.8 and autoclaved.

The cysteine and thioglycollic acid were autoclaved in neutral 1 per-cent solutions and kept under vaseline seal. The results reported in this paper were obtained with thioglycollic acid; no difference could be noticed between the action of the two thiol compounds.

The oxidized thiol compound used was a product obtained by the auto-oxidation of thioglycollic acid (Eastman), in the presence of air, with sodium nitroprusside as a catalyst (in dilute ammonia solution); this product will be referred to as “oxidized thioglycollic acid” although its exact nature is not known since it was not obtained in crystalline form.

The Bacteriostatic Action of Peptones and the Possibility of Correcting It by Means of Reduced Thiol Groups

It has been shown previously (3) that the growth of very small inocula of Pneumococcus in plain broth may occur when cysteine

hydrochloride or thioglycollic acid is added to the medium. Experiment 1 gives the minimum concentration of thioglycollic acid which is necessary for growth to occur.

Experiment 1—Varying amounts of thioglycollic acid were added to different portions of the same lot of plain broth (prepared 1 week previous to use), and tubes containing 5 cc. of these media received inocula varying from 0.1 cc. to 0.0000001 cc. of a plain broth culture of *Pneumococcus*. The cultures were incubated at 37°C. and final readings made after 48 hours incubation.

The results of Experiment 1 illustrate the fact that, in the plain broth under consideration, an inoculum of 0.1 cc. is necessary to obtain

TABLE I

The Influence of Thioglycollic Acid on the Growth of Small Inocula of Pneumococcus in Plain Broth (Peptone A)

Final concentration of thioglycollic acid (in per cent)	Inoculum (cc. of pneumococcus culture)							
	10 ⁻¹	10 ⁻²	10 ⁻³	10 ⁻⁴	10 ⁻⁵	10 ⁻⁶	10 ⁻⁷	10 ⁻⁸
0	+	—	—	—	—	—	—	—
0.001	+	+	+	—	—	—	—	—
0.002	+	+	+	—	—	—	—	—
0.005	+	+	+	+	+	+	+	—
0.01	+	+	+	+	+	+	+	—
0.02	+	+	+	+	+	+	+	+
0.05	+	+	+	+	+	+	+	—

The signs + and — refer to the presence or absence of growth after 48 hours incubation.

the growth of *Pneumococcus*. However, growth will occur even if the inoculum be only 0.000001 cc. in the presence of 0.005 per cent thioglycollic acid. It is apparent therefore that plain broth exerts a bacteriostatic effect on *Pneumococcus* and that a concentration of 0.005 per cent thioglycollic acid can overcome this effect.

The object of the next experiment was to determine whether the minimum concentration of thioglycollic acid required for the growth of small inocula of *Pneumococcus* would be affected by the kind of peptone used in the preparation of the medium.

Experiment 2.—Media were prepared from the same meat infusion but with different peptones, 2 per cent concentrations being used of either one of the four different commercial preparations of peptone designated as A, B, C, and D. Amounts of 5 cc. of these media were treated with different concentrations of thioglycollic acid, inoculated with 0.0001 cc. of pneumococcus culture and incubated at 37°C. Table II shows the results after 24 hours incubation.

The results of Experiment 2 indicate that the bacteriostatic effect of the broth varies greatly with the different peptones used in the preparation of the medium; this is evidence that a part, at least, of this bacteriostatic power is to be traced to the peptone.

TABLE II

The Effect of Using Different Commercial Peptones on the Growth of Pneumococcus in the Presence of Varying Concentration of Thioglycollic acid

Medium: meat infusion with	Final concentration of thioglycollic acid (in per cent)							
	0	0.0001	0.002	0.004	0.006	0.008	0.01	0.02
Peptone A.....	—	—	—	—	—	—	—	+
“ B.....	—	—	—	+	+	+	+	+
“ C.....	—	—	—	+	+	+	+	+
“ D.....	—	—	+	+	+	+	+	+

The signs + and — refer to the presence or absence of growth after 24 hours incubation.

The results also suggest that the amount of thioglycollic acid necessary for growth to occur might be used as a measure of the bacteriostatic action of the peptone.

The experiments that follow were all performed with peptone A which appears to have the most pronounced bacteriostatic power.

In order to demonstrate that thioglycollic acid can be used as a measure of the bacteriostatic action of the peptone, an attempt was made to establish a quantitative relationship between the amount of peptone added to the meat infusion and the concentration of thioglycollic acid required to overcome the bacteriostatic action of such media.

Experiment 3.—Media were prepared from the same meat infusion, but with concentrations of peptone (brand A) varying from 0.5 per cent to 4 per cent; these media were treated with varying concentrations of thioglycollic acid, inoculated

with 0.0001 cc. of pneumococcus culture (per 5 cc. of medium) and incubated at 37°C.

The results of Experiment 3 indicate that as the concentration of the peptone in the medium increases, the concentration of thioglycollic acid necessary for growth also increases. A more exact relationship will be obtained when, instead of the whole peptone, we use the isolated fraction responsible for the bacteriostatic action (see Experiment 8). This indicates that thioglycollic acid can be used to titrate the bacteriostatic power of the peptone.

TABLE III

The Relation between the Concentration of Peptone (Brand A) Used in the Preparation of the Broth, and the Concentration of Thioglycollic Acid Required for the Growth of Small Inocula of Pneumococcus

Concentration of peptone A (in per cent)	Final concentration of thioglycollic acid (in per cent)						
	0	0.001	0.002	0.004	0.006	0.008	0.01
4	—	—	—	—	—	—	+
2	—	—	—	—	+	+	+
1	—	—	—	+	+	+	+
0.5	—	+	+	+	+	+	+

The signs + and — refer to the presence and absence of growth after 48 hours incubation.

It has been suggested previously (3) that cysteine owes its beneficial action on the growth of *Pneumococcus* to its reducing power.

This suggestion will be confirmed by comparing the effect of the oxidized and reduced forms of thioglycollic acid on the growth of *Pneumococcus* in plain broth.

Experiment 4.—Different portions of plain broth prepared with 1 per cent of peptone A were treated with "reduced" and "oxidized thioglycollic acid." Amounts of 5 cc. of these media were seeded with inocula ranging from 0.1 to 0.00001 cc. of pneumococcus culture and incubated at 37°C.

The results of Experiment 4 indicate that, following oxidation, thioglycollic acid becomes unable to correct the bacteriostatic effect of the peptone; it may be noted, however, that "oxidized thioglycollic acid" is in no way toxic, as is shown by comparison with the control.

Thioglycollic acid therefore appears to owe its activity to the presence of reduced thiol groups in the molecule.

The Existence in Peptone A of Substances Which Are Bacteriostatic under Oxidizing Conditions and Not under Reducing Conditions

It has just been shown that the bacteriostatic power of peptone can be corrected by treatment with reduced thiol groups. It is also greatly affected by the conditions of oxidation and reduction under which the peptone solutions are kept. This can be shown by the five following examples.

Experiment 5.—a, b, c. Test tubes containing 4.5 cc. of meat infusion received 0.5 cc. of a 10 per cent peptone solution (at pH 7.8) which had been autoclaved.

In preparation *a*, the peptone solution was used *immediately after autoclaving*; in preparation *b*, it had been kept for 48 hours at room temperature under *acrobic conditions*; in preparation *c* it had been kept for 48 hours at room temperature under *vaseline seal*.

d. The peptone solution which had been kept aerobically for 48 hours (preparation *b*) was added to meat infusion and the mixture kept under vaseline seal for 2 weeks at room temperature.

e. A similar mixture (preparation *d*) which had been kept under vaseline seal for 2 weeks was now transferred to a flask and kept aerobically for 2 days.

Test tubes containing 5 cc. of the media (prepared as described under *a, b, c, d*, and *e*) were inoculated with 0.0001 cc. of pneumococcus culture and incubated at 37°C.

The results of Experiment 5 prove that peptone A used immediately after autoclaving, (preparation *a*), or kept constantly under anaerobic conditions (preparation *c*), exhibits only a very slight bacteriostatic power. On the other hand, the same peptone solution becomes markedly bacteriostatic following exposure to the air (preparation *b*). A peptone solution that has become bacteriostatic following exposure to the air loses its bacteriostatic effect by incubation under vaseline seal in the presence of meat infusion (preparation *d*); but again becomes bacteriostatic following reexposure to the air.

These facts, as well as the beneficial action of reduced thiol groups, point to the existence in peptone A of a substance which is bacteriostatic in the oxidized form, and not in the reduced form; it can be oxidized by atmospheric oxygen and reduced by -SH groups and meat infusion; this oxidation and reduction appears to be reversible.

In the course of these experiments, it was observed that the color of the peptone A varies with the different conditions under which the solution is kept. The solution is dark brown when taken out of the autoclave or when kept under vaseline seal. The color turns yellow

TABLE IV

The Growth of Pneumococcus in Plain Broth (Peptone A) in the Presence of Reduced and Oxidized Thioglycollic Acid

Medium	Inoculum (cc.)				
	10 ⁻¹	10 ⁻²	10 ⁻³	10 ⁻⁴	10 ⁻⁵
Plain broth	+	+	—	—	—
" " + 0.01 per cent reduced thioglycollic acid	+	+	+	+	+
Plain broth + 0.01 per cent oxidized thioglycollic acid	+	+	—	—	—

The signs + and — refer to the presence or absence of growth after 48 hours incubation.

TABLE V

Effect of Maintaining Peptone Solutions under Oxidizing and Reducing Conditions on the Bacteriostatic Effect of Media Prepared with Them

Media*	Final concentration of thioglycollic acid (in per cent)						
	0	0.0005	0.001	0.002	0.003	0.004	0.005
Preparation a	—	+	+	+	+	+	+
" b	—	—	—	—	—	—	+
" c	—	—	+	+	+	+	+
" d	—	+	+	+	+	+	+
" e	—	—	—	—	—	+	+

* The media referred to as preparations a, b, c, d, and e are described in the text.

The signs + and — refer to the presence or absence of growth after 48 hours incubation.

on the contrary under aerobic conditions. The yellow color could be changed again to the darker by treatment with thioglycollic acid or cysteine. In other words, the darker color corresponds to the reduced non-bacteriostatic form of the peptone solution, whereas the yellow color corresponds to the oxidized-bacteriostatic form.

It was tempting to suppose that the chromogenic fraction responsible for the change in color was associated with the bacteriostatic agent, and an attempt was made to fractionate peptone A on the basis of this assumption.

Isolation of the Fraction of Peptone A Containing the Bacteriostatic Substance

Different agents were used in an attempt to isolate the chromogenic fractions of peptone A (extraction and precipitation with HCl, NaOH, $(\text{NH}_4)_2\text{SO}_4$, copper salts, trichloroacetic acid, alcohol, acetone, ether). The best results were obtained with the following procedure.

200 gm. of peptone were dissolved in 1000 cc. of tap water and autoclaved. On the following day, the solution was treated with 15 cc. of concentrated hydrochloric acid. A heavy precipitate formed rapidly and was separated by filtration or centrifugalization. This dark green precipitate will be designated as fraction 1.

The filtrate (or supernatant) was made alkaline with 20 cc. of 40 per cent sodium hydroxide and 1000 cc. of acetone was added to the solution. A lighter precipitate of a greyish color separated and was designated as fraction 2.

A further amount of 1050 cc. acetone was added to the supernatant; a dark yellow (resin-like) material was seen to run down the walls of the container and could be separated in a separatory funnel. This will be designated as fraction 3.

After removal of this "yellow fraction," 3 liters of acetone were again added and a heavy white floculum was formed. This will be designated as fraction 4.

The remaining acetone solution was still slightly pigmented yellow; although it was found possible to separate this new pigment in very alkaline solution, no attempt was made to separate it on a large scale. The bulk of the solution was evaporated down and designated as fraction 5.

Each one of the 5 fractions was made up to 2 liters (corresponding therefore to a 10 per cent solution of peptone) and adjusted to pH 7.8. Fraction 1 appeared then as a dark green solution somewhat cloudy. Fraction 2 gave a greyish suspension, little soluble in water, and which settled somewhat on standing. Fraction 3 gave a perfectly clear, but very dark yellow solution. Fraction 4 gave a limpid solution with a very slight yellow tinge (due probably to a contamination with the preceding fraction). Fraction 5 gave a clear, slightly yellow solution.

5 cc. amounts of these different fractions were treated with 0.5 cc. of 1 per cent thioglycollic acid. Within half an hour (at room temperature), fraction 1 began to change color, turning first to a brown-yellow, then to a dark brownish color. The other fractions remained unchanged. It is therefore apparent that fraction 1 is responsible for the change in color observed in the peptone following oxidation and reduction.

It was also observed that following the addition of 0.1 cc. of a 3 per cent solution of potassium cyanide, fraction 1 turned whitish while the other fractions were not affected.

The object of this experiment was to determine which of the fractions were responsible for the bacteriostatic action of peptone A.

Experiment 6.—Media were prepared by the addition of 0.5 cc. of the different fractions and of whole peptone (10 per cent solution) to 4.5 cc. of meat infusion. These media were treated with different concentration of thioglycollic acid and inoculated with 0.0001 cc. of a pneumococcus culture.

TABLE VI

The Bacteriostatic Effect on Pneumococcus of Media Prepared by the Addition to Meat Infusion of Different Fractions of Peptone A

Medium containing	Final concentration of thioglycollic acid (per cent)					
	0	0.001	0.002	0.003	0.004	0.005
Fraction 1.....	—	—	—	—	—	—
" 2.....	+	+	+	+	+	+
" 3.....	+	+	+	+	+	+
" 4.....	+	+	+	+	+	+
" 5.....	—	—	—	—	—	—
Whole peptone.....	—	—	—	—	+	+

The signs + and — refer to the presence or absence of growth after 48 hours incubation.

It is apparent from the results of Experiment 6 that fractions 2, 3 and 4 of peptone A can support the growth of *Pneumococcus* when added to meat infusion—and do not exhibit any bacteriostatic effect. The absence of growth with fractions 1 and 5 may be attributed to the lack of some factor essential to growth or to the bacteriostatic action of these fractions. This question is the subject of the following experiment.

Experiment 7.—A medium was prepared by the addition of 15 cc. of fraction 4 to 135 cc. of meat infusion. To tubes containing 5 cc. amounts of this medium was added 0.5 cc. of peptone solution (10 per cent), of fraction 1 or of fraction 5 along with different concentrations of thioglycollic acid. The tubes were then inoculated with 0.0001 cc. of pneumococcus culture and incubated at 37°C.

The results of Experiment 7 demonstrate that practically all the bacteriostatic action of the peptone is due to fraction 1 and only to it.

In all these experiments, thioglycollic has been used as a reagent for titrating the bacteriostatic action of the materials under consideration. In fact, it has been found in Experiment 3 that the amount of thioglycollic acid required to initiate growth of small inocula increases with the concentration of peptone in the medium. The subject of Experiment 8 was to determine whether a more exact relationship could be obtained between the amount of fraction 1 added to the medium and that of thioglycollic acid required to overcome its bacteriostatic action.

Experiment 8.—Attention must be called to two modifications introduced in the experimental procedure. Meat infusion is known to possess a high reducing

TABLE VII

The Bacteriostatic Action of Fraction 1 of Peptone A

Medium: meat infusion with	Concentration of thioglycollic acid (per cent)						
	0	0.0004	0.002	0.005	0.01	0.02	0.05
Fraction 4.....	+	+	+	+	+	+	+
“ 4 + fraction 1.....	—	—	—	+	+	+	+
“ 4 + fraction 5.....	—	+	+	+	+	+	+
Whole peptone.....	—	—	—	+	+	+	+

The signs + and — refer to the presence or absence of growth after 48 hours incubation.

capacity and it is likely that it would thus interfere with the establishment of the quantitative relationship between fraction 1 and thioglycollic acid. Tryptophane broth on the contrary has very little reducing capacity; it can support the growth of *Pneumococcus* when added to fraction 3 of peptone. It was therefore used instead of meat infusion in this experiment.

The other modification was devised in view of the fact that the fraction 1, after being reduced by thioglycollic acid, may be reoxidized by atmospheric oxygen. This was prevented by incubating the culture under vaseline seal.

Varying amounts of fraction 1 were added to test tubes containing 4.5 cc. of medium consisting of tryptophane broth and fraction 3; these media were treated with different concentrations of thioglycollic acid, inoculated with 0.0001 cc. of pneumococcus culture and incubated under vaseline seal.

Table VIII brings out the close proportionality between the concentration of fraction 1 in the medium and the amount of thioglycollic

TABLE VIII

Titration of the Bacteriostatic Fraction of Peptone A by Means of Thioglycollic Acid

Amount of fraction 1 added to the medium (in per cent)	Final concentration of thioglycollic acid (in per cent)						
	0	0.0005	0.001	0.002	0.005	0.01	0.02
0	+	+	+	+	+	+	+
0.2	—	+	+	+	+	+	+
0.5	—	—	—	—	+	+	+
1.0	—	—	—	—	—	+	+
2.0	—	—	—	—	—	—	+

The signs + and — refer to the presence or absence of growth after 48 hours incubation.

TABLE IX

The Effect of Potassium Cyanide on the Bacteriostatic Properties of the Fraction 1 of Peptone A

Medium: meat infusion with	Final concentration of thioglycollic acid (in per cent)				
	0.0005	0.002	0.005	0.01	0.02
Fraction 4.....	+	+	+	+	+
KCN + fraction 4.....	+	+	+	+	+
Fraction 1 + fraction 4.....	—	—	—	+	+
KCN + fraction 1 + fraction 4.....	+	+	+	+	+

TABLE X

The Growth of Pneumococcus in Media Prepared with Whole Peptone and "Purified Peptone"

Materials added to meat infusion	Final concentration of thioglycollic acid (in per cent)						
	0	0.0001	0.0002	0.005	0.01	0.02	0.05
Whole peptone.....	—	—	—	—	—	—	+
Purified peptone.....	—	+	+	+	+	+	+
Fraction 1.....	—	—	—	—	—	—	—
Purified peptone + fraction 1.....	—	—	—	—	—	—	—

The signs + and — refer to the presence or absence of growth after 48 hours incubation.

acid required for growth to develop. This relation, however, does not apply when very small concentrations are used.

It has been mentioned previously that, when treated with potassium cyanide, the green pigment rapidly becomes whitish. Experiment 9 demonstrates that fraction 1 loses at the same time its bacteriostatic properties.

Experiment 9.—A medium was prepared by the addition of 10 per cent of fraction 4 to meat infusion. Test tubes containing 5 cc. of this medium received 0.5 cc. of fraction 1, or 0.02 cc. of 3 per cent KCN, or both. They were then treated with different concentrations of thioglycollic acid, inoculated with 0.0001 cc. of pneumococcus culture and incubated at 37°C.

The results of Experiment 9 show that the bacteriostatic properties of fraction 1 can be removed by treatment with potassium cyanide.

The preceding experiments suggest that a solution of peptone A from which the green bacteriostatic fraction has been removed should supply a satisfactory medium when added to meat infusion.

Experiment 10.—A 10 per cent solution of peptone A was treated with the proper amount of concentrated HCl to precipitate fraction 1. This fraction was then made up to the original volume of the peptone solution and adjusted to pH 7.8.

The peptone solution from which fraction 1 had been removed appeared as a yellow fluid which was also adjusted to pH 7.8. This second fraction will be designated as "purified peptone."

These two solutions were added in amounts of 0.5 cc. to 4.5 cc. of meat infusion as described in Table X and the media treated with different concentrations of thioglycollic acid. The tubes were inoculated as usual with 0.0001 cc. of pneumococcus culture and incubated at 37°C.

The results of Experiment 10 demonstrate that by acid precipitation, one can remove almost completely the bacteriostatic properties of a solution of the commercial peptone A.

DISCUSSION

The facts recorded in this article seem to warrant the conclusion that there exists in commercial peptones a substance (or substances) which may exert a bacteriostatic action on the growth of certain organisms. Its action in plain broth is well demonstrated by the lack

of growth of small inocula of *Pneumococcus* under ordinary conditions. When, on the contrary, the same medium is prepared with peptones from which the bacteriostatic substance has been removed, or when the bacteriostatic effect has been corrected by reduction, inocula consisting of only one or a very few cells are sufficient to initiate growth.

The bacteriostatic substance is heat stable and, in the brand of peptone which has been studied, it is associated with a certain fraction obtained by acid precipitation; this fraction is pigmented, and changes color upon oxidation and reduction; the bacteriostatic property of the same peptone fraction is also controlled by conditions of oxidation and reduction. But we have no other evidence that the chromogenic substance and the bacteriostatic substance are one and the same thing.

Concerning the mode of action of this substance, we have at least an excellent clue; it is bacteriostatic only under oxidizing conditions, not at all under reducing conditions. In this respect, it behaves similarly to some dyes previously studied (indophenols, methylene blue, gallocyanine,¹ which are bacteriostatic in the oxidized form, but not in the reduced form (3).

Methylene blue has been found to be bacteriostatic for *Pneumococcus* in concentrations as low as 0.00003 molar. There is as yet no direct way of measuring at what minimum concentration the bacteriostatic substance of peptone exerts its activity, but some conception can be gained by an indirect method. As already shown, there exists a certain quantitative relationship between the amount of bacteriostatic substance and the concentration of thioglycollic acid which can overcome its action. In certain experiments, it has been sufficient to add as little as 0.0003 per cent of this thiol compound to allow the growth of an inoculum which would not have otherwise developed; the molecular weight of thioglycollic acid being 92, one sees that the molar concentration is of the same order as that found in the case of methylene blue. Expressed in other terms, this means that the addition of 0.000003 gm. of thioglycollic acid per cubic centimeter of medium may determine whether growth will take place or not. These concentrations are of the order found in catalytic reactions, and one may well wonder whether the bacteriostatic substance of the peptone

¹ The data concerning the effect of gallocyanine have not yet been published.

does not act as anticatalyst in some phase of the metabolism of the cell.

Whatever the mechanism of its action may be, this substance is active in extremely small amounts. We have evidence of organisms which are much more sensitive to it than the ordinary strains of *Pneumococcus*. It will be worth while to study the rôle of such substances in determining whether a bacterial species is "aerobic" or "anaerobic," whether it is "easy" or "hard" to grow.

The growth of large inocula of *Pneumococcus* in plain broth, as compared with the lack of development of smaller inocula, is now readily understood. It has been shown (1, 2) that pneumococcus cells, when in the presence of certain metabolites, give rise to a very actively reducing system; this system is able to reduce (and therefore inactivate) the bacteriostatic substance of the peptone and thus renders the medium suitable for the development of growth.

The significance of the facts presented in this article may well transcend their importance for the growth of *Pneumococcus* in artificial media. One wonders whether there do not exist in tissues mechanisms similar to the one described, which would render impossible the multiplication of bacteria under normal conditions of oxidation. If, however, local reducing conditions were to develop, is it not possible that bacterial invasion of the tissues would then be facilitated? It is well to remember, in this respect, that the peptones under consideration were probably prepared from materials of animal origin. In fact, tryptic digest broth and peptic digest broth prepared from beef were also found to exhibit some bacteriostatic properties, but this action was demonstrable only under aerobic conditions.

SUMMARY

There are present in commercial peptones substances which exhibit bacteriostatic properties for certain bacterial species. These substances are bacteriostatic in the oxidized form, but not in the reduced form. Their bacteriostatic action can be overcome, and their concentration titrated, by the addition of reduced thiol compounds to the media in which they are present.

Different brands of peptone differ greatly in the amount of bacteriostatic substances they contain; these differences account, in part at

least, for the fact that media prepared from the same meat infusion, but with different kinds of peptone, vary in their ability to support bacterial growth.

The bacteriostatic fraction of a certain peptone solution can be completely removed by precipitation with acid and acetone. A peptone which has thus been purified becomes capable of supporting the growth of very small inocula of *Pneumococcus*.

The significance of the sensitiveness of certain bacterial species to substances which are bacteriostatic in the oxidized but not in the reduced form is considered with reference to (a) the mechanism of bacteriostasis, (b) the growth of bacterial species in artificial media, (c) the problem of infection.

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EXPERIMENTS ON ANAPHYLAXIS TO AZOPROTEINS

THIRD PAPER

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In preceding papers (1, 2) it has been shown to be possible to induce anaphylaxis in guinea pigs by injecting azoproteins, namely, horse serum coupled with diazotized *p*-arsanilic acid. Animals treated in this way become sensitive to azoproteins containing the same azocomponent but another sort of protein; thus the reactions obviously depend upon the arsanilic acid group.

These results have been confirmed by Meyer and Alexander (3) who used also the method of passive sensitization, and by Klopstock and Selter (4). Some of the observations of these workers will be discussed later. Another investigation on the subject has been made recently by Tillett, Avery and Goebel (5). These authors employed as antigens azoproteins prepared by combining globulin or albumin with diazotized glucosides.

The present study was undertaken with the purpose of investigating the specificity of the anaphylactic reactions and of gaining further information on the inhibition phenomenon produced by simple chemical substances (2). In order to test the specificity of anaphylaxis to azoproteins, antigens were selected which differed only in the steric configuration of the specifically reacting groups. The fact that antigens containing sterically isomeric groups are serologically different has already been shown by means of the precipitin reaction by Landsteiner and van der Scheer (6, 7) for *l*- and *d*-phenyl (*p*-aminobenzoylamino) acetic acids and *l*-, *d*-, and *m*-tartaric acids. Similar results were obtained by Avery and Goebel (8, 9) who used as antigens glucosides containing glucose or galactose.

EXPERIMENTAL

Specificity of the Anaphylactic Reaction.—For the following experiments antigens were used containing the radicals of the *d*- and *l*-tartaric acids. These were prepared by coupling horse serum with *d*- and *l*-*p*-aminotartranilic acid in the manner previously described (7).

For the sensitization guinea pigs were injected intraperitoneally three times at weekly intervals with a suspension of 1 cc. of the antigens containing about 5 per cent protein. The animals weighed 210 to 250 gm.; the injections were well tolerated.

The solutions used for the reinjection were prepared in the same manner as the sensitizing antigens except that chicken serum was employed instead of horse serum and that after precipitation with acid, the azoproteins, without treatment with alcohol, were brought in solution with the aid of sodium carbonate; the solutions were made isotonic and adjusted to litmus neutrality. The stock solutions of the antigen were brought to a protein content of 3.5 per cent. The test injections were made intravenously 3 weeks after the last administration of the sensitizing dose, with 1 cc. of various dilutions or a larger volume of the concentrated antigen. At the time of the test the weight of the animals was about 400 gm. The results of an experiment in which the specificity of the reaction was tested are given in Table I.

From Table I it is seen that, with one possible exception, the sensitization succeeded regularly; one animal showed only slight symptoms. The quantity sufficient for inducing shock was as low as 0.35 to 0.7 mg. The symptoms were in all cases typical of anaphylactic shock, and in the animals which died, the lungs were distended and the heart was beating. The reactions were strikingly specific since an injection of about 50 to 100 minimal lethal doses was innocuous for the animals sensitized to the heterologous antigen, apart from a drop in temperature which generally did not exceed 1°. In this respect the results are in full agreement with those reported by Tillett, Avery, and Goebel (5).

A further proof of the specificity of the reactions was furnished by reinjecting, on the following day, some of the animals which had received a dose of the heterologous antigen without showing symptoms of anaphylactic shock. Such animals, with one exception, reacted to

a subsequent injection of the same quantity of the homologous antigen, although there was evidence of some protection (Table II).

TABLE I¹
Animals Sensitized with d-Antigen

Tested with d-antigen				Tested with l-antigen			
Guinea pig No.	Quantity of azo-protein injected in mg.	Subsequent change in body temperature	Result, symptoms	Guinea pig No.	Quantity of azo-protein injected in mg.	Subsequent change in body temperature	Result, symptoms
		°C.				°C.	
1	70		† 4 min.	14	70	-0.7	Negative
2	35		† 3 "	15	70	-0.4	"
3	35	-4.3	Severe	16	35	-1.5	Slight
4	35		† 3 min.	17	35	-0.7	Negative
5	35		† 3 "	18	17.5	-0.4	"
6	35		† 3 "	19	8.8	-0.9	"
7	35		† 5 "	20	0.7	+0.9	"
8	17.5	-3.3	Severe				
9	17.5		† 4 min.				
10	8.8		† 3 "				
11	1.5		† 4 "				
12	0.7	-2.3	Very severe				
13	0.35	-0.6	Moderate				

Animals Sensitized with l-Antigen

Tested with d-antigen				Tested with l-antigen			
Guinea pig No.	Quantity of azo-protein injected in mg.	Subsequent change in body temperature	Result, symptoms	Guinea pig No.	Quantity of azo-protein injected in mg.	Subsequent change in body temperature	Result, symptoms
		°C.				°C.	
21	70	+1.0	Negative	26	70		† 4 min.
22	35	-1.1	"	27	35	-2.8	Slight
23	8.8	-1.9	"	28	35		† 3 min.
24	3.0	-0.5	"	29	8.8		† 3 "
25	0.7	-0.6	"	30	3.0		† 5 "
				31	0.7		† 5 "
				32	0.35	-2.4	Severe
				33	0.18	-1.3	Slight

¹ The designations correspond to those in the previous paper (7).

† Death of animal.

Guinea pigs which were sensitized with only one injection of 1 cc. of the antigen showed the same degree of sensitivity as those in the

TABLE II
Reinjection Experiments
Animals Sensitized with d-Antigen

Guinea pig No.	Quantity of l-antigen injected in mg.	Result, symptoms	Quantity of d-antigen injected in mg.	Subsequent change in body temperature	Result, symptoms
				°C.	
17	35	Negative	35	-1.8	Negative
18	17.5	"	17.5		† 5 min.
19	8.8	"	8.8	-2.5	Severe
20	0.7	"	0.7		† 8 min.

Animals Sensitized with l-Antigen

Guinea pig No.	Quantity of d-antigen injected in mg.	Result, symptoms	Quantity of l-antigen injected in mg.	Subsequent change in body temperature	Result, symptoms
				°C.	
21	70	Negative	70		† 8 min.
22	35	"	35		† over night
24	3	"	3	-2.2	Severe
25	0.7	"	0.7		† 5 min.

TABLE III

Guinea pigs weighing 200 to 220 gm. were given one intraperitoneal injection of d-antigen: reinjection with shocking antigen at the end of 23 days when the animal weighed about 300 gm.

Guinea pig No.	Quantity of d-antigen injected in mg.	Subsequent change in body temperature	Result, symptoms
		°C.	
34	3		† 3 min.
35	0.7		† 4 "
36	0.7		† 5 "
37	0.35	-0.7	Moderate to severe

experiment reported in Table I since they also succumbed to a shocking dose of 0.7 mg. An example is given in Table III.

The above experiments furnished hardly any evidence of the inhibition of the anaphylactic reaction by the administration of large doses of the shocking antigen, which was observed by Klopstock and Selter (4). However there was some indication of the zone phenomenon in experiments (Table IV) in which animals were sensitized with an antigen made by coupling beef serum with diazotized arsanilic acid as was done by Klopstock and Selter.

It is seen from Table IV that only one of three animals was killed

TABLE IV

Eleven guinea pigs were sensitized by one subcutaneous injection of atoxyl beef antigen (1 cc. = 16.6 mg.) which was purified by means of acid and alcohol; the reinjection with atoxyl chicken antigen prepared in the same manner as the beef antigen was made 16 days after the sensitization. The reinjection was made intravenously in a volume of 1 cc.

Guinea pig No.	Quantity of chicken antigen injected in mg.	Subsequent change in body temperature	Result, symptoms
		*C.	
38	6	-6.2	Very sick
39	6	-1.1	Negative
40	6		† 5 min.
41	1.5	-1.8	Slight to moderate
42	1.5		† 4 min.
43	1.5		† 7 "
44	1.5		† 7 "
45	1.5		† 6 "
46	0.5	-1.3	Slight to moderate
47	0.5		† 5 min.
48	0.5		† 6 "

by the largest dose employed while four out of five succumbed to a dose four times smaller.

Sensitization with Antigens Prepared According to the Method of Klopstock and Selter.—Whilst in the first experiments on anaphylaxis to azoproteins, the antigens used for sensitization were isolated after coupling in alkaline solution by precipitation with acid, Klopstock and Selter sensitized guinea pigs by injecting guinea pig serum to which they added neutralized diazosolutions. The reinjections were made with azoproteins prepared from guinea pig serum according to the older

method. In the experiment to be described (Table V) the procedure of Klopstock and Selter was followed.

The results as judged from the reinjection with the chicken serum preparation confirmed in a general way those of Klopstock and Selter except that the sensitization did not succeed regularly. They differed in that most of the animals tested did not react to the guinea pig serum

TABLE V

Eighteen guinea pigs were sensitized by a subcutaneous injection of 1 cc. of a solution made by adding two volumes of 1 per cent neutralized solution of diazotized *p*-arsanilic acid to one volume of fresh guinea pig serum (4). The solution stood overnight in the ice box before injections were made. The reinjections were made after an interval of 33 days.

Reinjection with chicken <i>p</i> -arsanilic acid antigen (1 cc. = 18.6 mg. protein)				Reinjection with guinea pig <i>p</i> -arsanilic acid antigen (1 cc. = 23.8 mg. protein)			
Guinea pig No.	Quantity of antigen in mg.	Subsequent change in body temperature	Result, symptoms	Guinea pig No.	Quantity of antigen in mg.	Subsequent change in body temperature	Result, symptoms
		°C.				°C.	
49	9.0		† 4 min.	59	12	-1.2	Negative
50	9.0	-1.3	Slight	60	12	-0.4	"
51	5.0		† 3 min.	61	5	-1.5	Slight to moderate
52	5.0		† 20 "	62	5		† 4 min.
53	5.0	-0.3	Negative	63	5	-0.2	Negative
54	5.0		† 20 min.				
55	5.0	-1.0	Negative				
56	1.5	-0.3	"	64	1.5	-0.6	"
57	1.5		† 6 min.	65	1.5	-1.8	"
58	1.5	-0.8	Negative	66	1.5	-0.6	"

preparation. No attempt was made to inquire into the cause of this discrepancy.

From their observations Klopstock and Selter conclude that for the sensitization and the production of antibodies, as well as for the reactions *in vitro*, it is not necessary to have a chemical combination of the azocomponents with protein but that it suffices to use simple "mixtures" of diazocompounds and protein. They stress the view that the diazocompounds would, by themselves, act as antigens and the proteins only enhance the antigenic activity which is inherent in

the simple substances (4, 10, 11). However, as has been shown by Heidelberger and Kendall (12) and one of the present writers (13), there undoubtedly takes place a combination of the diazocompounds with proteins also in neutral solution under the conditions of the method of Klopstock and Selter. Consequently their procedure does not involve a new principle but must be looked upon as a modification of the older method of preparing azoproteins. It is true that in a footnote to their last publication¹ the authors make a statement which may be interpreted as an admission that they dealt not with mixtures but with chemical combinations. As for those instances in which Klopstock and Selter succeeded in sensitizing with diazocompounds alone, it can be assumed that these substances combined with the proteins of the animal injected, so that in this case also the immunization is probably attributable to an azoprotein. Indeed it has been shown that animals can be immunized by azoproteins the protein part of which is derived from their own species (14).

The phenomenon observed by Klopstock and Selter, that guinea pigs sensitized with diazosolutions alone exhibit skin reactions on intradermal injections of the same diazocompound, may be due to a special mechanism if further study should show that the skin reactions can be induced in this way only and not by sensitization with azoproteins. One may suppose either that the chemical combinations formed in the body on the injection of diazosolutions are different from those prepared *in vitro* or that the sensitization is brought about by the direct action on tissues (skin) by the diazocompound as such. Even in the latter case, because of the ease with which diazosolutions combine with proteins it would not be justifiable to draw conclusions, from the experiments discussed, upon the possibility of sensitization with simple chemical substances in general, particularly those which do not readily form compounds with proteins.

Inhibition of Anaphylactic Shock by Simple Substances.—In the experiments of Landsteiner (2) a peculiar phenomenon was noticed, *i.e.* shock could be prevented by injecting sensitized animals with simple azocompounds containing the same specific group as the sensitizing antigen. The substances used were compounds prepared

¹ See (4), page 465.

by coupling diazotized arsanilic acid with tyrosine or *p*-oxybenzoic acid. Analogous results were described by Klopstock and Selter (4) with the sodium salts of *p*-arsanilic acid (atoxyl) and *m*-aminobenzenesulfonic acid. K. Meyer did not succeed in obtaining antianaphylaxis by injecting atoxyl into animals sensitized to azoproteins prepared from *p*-arsanilic acid.

Similar experiments were carried out by Tillett, Avery, and Goebel (5) with guinea pigs sensitized to azoproteins containing glucosides. When these animals were injected with uncombined glucoside immediately prior to the administration of the antigen, shock could be prevented. If, however, the shocking injection was given 2 hours later protection was no longer demonstrable. Consequently the authors raise the question as to the mechanism of this inhibitory effect which, indeed, can hardly be looked upon as a desensitization on account of its transitory nature.

Our present experiments were carried out with a series of animals sensitized with antigens prepared from *d*- and *l*-*p*-aminotartranilic acid and another series sensitized to azoproteins prepared from *p*-arsanilic acid.

Guinea pigs weighing 200 to 250 gm. were sensitized as in the previous experiment (see Table I) and were tested 3 weeks after the last injection. At various intervals before the administration of the shocking homologous antigen the animals were injected with solutions of an azocompound made by coupling resorcinol with diazotized *d*- and *l*-*p*-aminotartranilic acids. The products are designated as *d*-T.R. and *l*-T.R., respectively.

These substances were prepared as follows: 480 mg. *d*- or *l*-*p*-aminotartranilic acid were diazotized in the usual way (7, page 410) and coupled with 110 mg. resorcinol. The dye formed was precipitated with the aid of dilute hydrochloric acid, the precipitate washed in acidulated water and dried.

A 1 per cent solution of the dye was made in a 0.9 per cent salt solution by adding dilute sodium hydroxide, and the solution was adjusted to neutrality or faint alkalinity. 1 cc. of various dilutions was injected intravenously. The shocking antigen was injected in a quantity of 7 mg. (1 cc. of a 0.7 per cent dilution) which corresponds to 5 to 10 minimal lethal doses (see Table I). At the time of the tests the weight of the guinea pigs was about 400 gm.

The experiments (Table VI) demonstrate that with one exception in which the animal showed severe anaphylactic symptoms, the guinea pigs previously injected with the heterologous azodye died in typical

TABLE VI
Inhibition by the Injection of Azodyes
Animals Sensitized with d-Antigen

Guinea pig No.	d-T.R. mg.	Interval between injections in hours	Result and symptoms after injection of d-antigen	Subsequent change in body temperature	Guinea pig No.	L-T.R. mg.	Interval between injections in hours	Result and symptoms after injection of d-antigen	Subsequent change in body temperature
				°C.					°C.
67	10	1	Weakness	-4.5	77	10	1	† 4 min.	
68	10	18	Cough, slight weakness	-1.1	78	10	18	† 4 "	
					79	5	1	† 4 "	
69	10	18	Dyspnea spasms	-1.2	80	5	2½	† 4 "	
70	5	1½	Weakness	-0.2	81	5	2½	† 5 "	
71	5	2	Negative	+0.7	82	5	3½	† 4 "	
72	5	2½	Slight	-0.7	83	5	24	† 5 "	
73	5	2½	Negative	-0.3	84	2.5	1	† 5 "	
74	5	24	Cough	-4.0	85	2.5	2½	Few convulsions, very sick	-3.6
75			Weakness						
75	2.5	1	Somewhat sick, weakness	-1.3					
76	2.5	2	Negative	+0.6					

Animals Sensitized with l-Antigen

Guinea pig No.	d-T.R. mg.	Interval between injections in hours	Result and symptoms after injection of l-antigen	Guinea pig No.	L-T.R. mg.	Interval between injections in hours	Result and symptoms after injection of l-antigen	Subsequent change in temperature
								°C.
86	10	16	† 4 min.	96	10	16	Slight, weak	-2.8
87	5	2½	† 4 "	97	10	16	Negative	-1.0
88	5	3	† 4 "	98	5	2½	Negative	-0.2
89	5	3	† 4 "	99	5	3	† 5 min.	
90	5	3	† 5 "	100	5	3	Somewhat weak	-1.0
91	5	3	† 2 "					
92	5	3	† 5 "	101	5	3	Weakness	-1.9
93	5	3	† 4 "	102	5	3	Slight, weak	-0.5
94	5	3½	† 4 "	103	5	3	Weak	-1.8
95	5	4	† 6 "	104	5	3	Negative	-0.4
				105	5	3½	Slight, weak	-1.6
				106	5	3½	Slight, weak	-1.6

acute shock, whilst all animals but one, injected with quantities of 2.5 to 10 mg. of the homologous dye, survived. The surviving animals exhibited but rarely typical anaphylactic symptoms as spasms or cough, although many appeared sick. With higher doses of the dye the protection was still evident even when the injection of the antigen was made the following day.

Another batch of animals was sensitized passively by injecting a potent precipitating immune serum produced in a rabbit by immunization with azoprotein made from horse serum and diazotized *p*-arsanilic acid.² On injecting intraperitoneally guinea pigs weighing about 300 gm. with 0.3 cc. of this immune serum, the animals proved to be sensitive to an azoprotein prepared from diazotized *p*-arsanilic acid and chicken serum. The minimal lethal dose was regularly found to be 0.5 mg. In the tests presented in Table VII the animals were passively sensitized with 0.3 cc. of the immune serum. The substance tested for inhibition was a product of coupling diazotized *p*-arsanilic acid and tyrosine (2). This was injected intravenously in a volume of 0.5 cc. at stated intervals prior to the administration of the antigen or in a mixture with antigen (indicated as "0" in Table VII). The antigen was employed in a quantity corresponding to two minimal lethal doses.

From Table VII it is seen that the animals were protected from lethal shock by quantities of 2.5 to 1.25 mg. of the dye regardless of the time interval between the two injections. These animals showed either no symptoms or became somewhat weak. In two animals only were slight convulsions or coughing observed. With 0.6 and 0.3 mg. of the dye the results were irregular but again the outcome appeared not to depend on the time elapsed after the injection. A still smaller dose (0.15 mg.) failed to prevent shock also when the dye was injected simultaneously with the antigen.

A few experiments with solutions of sodium *p*-arsanilate seemed to indicate that also this substance has an inhibitory effect upon the anaphylactic reaction but considerably larger quantities were used than of the azosubstance.

The protection described can be explained in two ways, either by assuming that this effect is similar to the inhibition of the precipitin

² For the method see (15).

reaction *in vitro* by simple substances containing the specific group (16), or that the mechanism is analogous to the well known desensitiza-

TABLE VII

Inhibition by the Injection of Azodye

Animals passively sensitized with 0.3 cc. immune serum; the next day injection of the compound of *p*-arsanilic acid and tyrosin, followed by the administration of 1 mg. of the shocking antigen.

Guinea pig No.	Atoxyl-tyrosine in mg.	Interval between injection of dye and antigen in hours	Shocking antigen	Result and symptoms after injection of shocking antigen	Subsequent change in body temperature
					°C.
107	2.5	3	1 mg.	Somewhat weak	-1.6
108	2.5	18	1 "	Few coughs, somewhat weak	-1.6
109	2.5	18	1 "	Negative	+0.55
110	1.25	0	1 "	Vigorous scratching	-1.9
111	1.25	0	1 "	Negative	-1.55
112	1.25	3	1 "	"	-0.75
113	1.25	3	1 "	"	-1.8
114	1.25	18	1 "	Spasms	-2.4
115	0.6	0	1 "	† 4 min.	
116	0.6	0	1 "	Convulsions, weak	-2.3
117	0.6	3	1 "	Spasms	-1.55
118	0.6	3	1 "	Negative	-1.25
119	0.6	3	1 "	"	0
120	0.6	18	1 "	† 4 min.	
121	0.3	0	1 "	Somewhat weak	-1.1
122	0.3	0	1 "	† 4 min.	
123	0.3	3	1 "	Dyspnea, somewhat weak	-1.45
124	0.3	3	1 "	Severe	-8.85
125	0.3	3	1 "	† 6 min.	
126	0.3	4	1 "	Negative	-0.9
127	0.15	0	1 "	† 4 min.	
128	0.15	0	1 "	† 4 "	
129	0.15	3	1 "	Very severe, almost dying	-2.4
130			0.5 "	† 5 min.	
131			0.5 "	† 6 "	
132			0.5 "	† 3 "	
133			0.5 "	† 4 "	
134			0.5 "	† 4 "	
135			0.5 "	† 3 "	
136			0.25 "	Slight to moderate	-3.0

tion by small quantities of antigen. On the first assumption one would expect protection to diminish with the elimination of the inhibiting substance from the blood stream. Actually the elimination takes place rather quickly since soon after the injection the urine is distinctly colored. Further evidence was gained from an examination of the color of the serum of guinea pigs after intravenous injection of the dye and from an estimation of the dye in the serum by inhibition of the precipitin reaction. From the few tests made it appeared that a considerable part of the azodye (about half) was already eliminated within the first hour after the injection.

On the other hand protection was still demonstrable on the day following the administration of the dye, and furthermore, in the experiments of Table VII there was no noticeable difference in the results whether the inhibiting substance was injected simultaneously with the antigen or 3 hours afterwards. Consequently one can conclude that the effect is not due simply to the presence in the circulation of the substances tested but to a desensitizing action upon the tissues. This view is corroborated by the observation that frequently the injection of homologous azodye into sensitized animals was followed by a significant drop in temperature and in a number of cases even by anaphylactic shock (17), but in some series of experiments this result could not be duplicated.

It is possible, however, that there are other instances in which protection is brought about by the same mechanism as inhibition *in vitro*. This is suggested by the results of Tillett, Avery, and Goebel, who noticed that their glucosides prevented shock only when injected just prior to the antigen, but not after an interval of 2 hours. The apparent discrepancy between their results and the present ones can probably be attributed to differences in the chemical nature of the substances used.

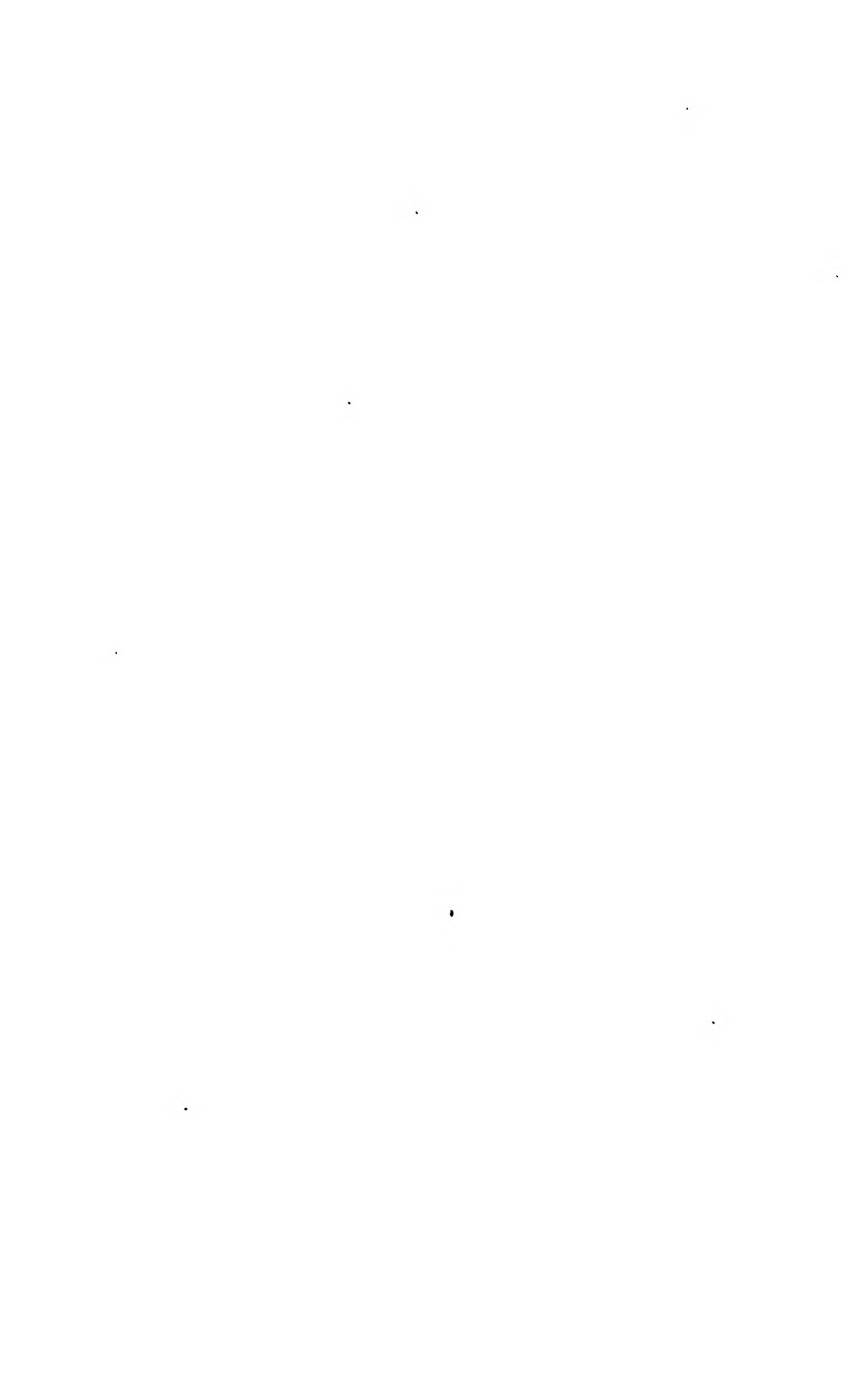
SUMMARY

Experiments with azoproteins containing stereo-chemical isomeric groups of *d*- and *l*-tartaric acid showed well marked specificity of the anaphylactic reaction to these antigens, in conformity with the results of precipitin tests.

Shock in these animals could be prevented by injection of azodye containing the specific groups. This phenomenon is ascribed to a desensitization.

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SURVIVAL AND MULTIPLICATION OF THE VIRUS OF POLIOMYELITIS IN VITRO

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The subject of the artificial cultivation of filterable viruses has again brought into prominence the experiments of Flexner and Noguchi (1) and their followers with the so-called globoid bodies of poliomyelitis. The present paper deals with an attempt to repeat the results of the studies of Flexner and Noguchi and it is therefore desirable to present in brief form the results of the earlier work.

The original paper of Flexner and Noguchi (1) described the globoid bodies as minute, formed structures, cultivated by specially devised methods from tissues of the central nervous system from both human and experimental sources. The tissues were either fresh or glycerolated, unfiltered or filtered through Berkefeld V or N candles.

Methods of Cultivation.—The culture medium consisted essentially of human ascitic fluid to which had been added a fragment of sterile fresh tissue, usually rabbit's kidney. The liquid was covered with a deep layer of sterile paraffin oil and of the two sets of cultures, one was placed in an anaerobic jar and the other kept outside. All tubes were incubated at 37°C.

In the tubes kept outside the jar, it was observed after 5 days that about the fragments of tissue an opalescence appeared which could be gradually diffused through the tube by gentle shaking. After another period of 3 to 5 days, the opalescence extended into the upper portion of the medium, but at the expiration of 10 to 12 days the diffuse but slight opacity began to diminish, and irregular particles formed and slowly fell to the bottom of the tube. In control tubes, either no change was observed, or a slight granular precipitate covered the tissue; and, while in tubes in which growth had been noted the opalescence increased in extent, in the control series the precipitate gathered progressively about the tissue fragments. In the corresponding tubes kept in an anaerobic jar, a similar opalescent growth was seen, although it was slighter than that just described and required a somewhat longer time to reach its full development.

For the demonstration of colony formation, a semisolid medium was used which comprised ascitic fluid and sterile rabbit kidney, to which sufficient 2 per cent

agar had been added to produce a solid mixture. This medium was not successful for initial growths, but after about the third generation of growth in fluid medium, transfer could be effected to the solid medium. In this, growth was noted after several days as a diffuse opalescence which first appeared about the tissue fragments and gradually aggregated into visible, minute colonies. Later, the opalescence rose in the medium until it reached about 3 cm. below the surface, the upper limit of growth being sharply demarcated.

In the original paper, mention was made of the fact that initial growth could be secured in a medium from which either rabbit kidney or ascitic fluid had been eliminated, the former being replaced by using a somewhat larger fragment of poliomyelitic brain or the latter by adding brain tissue extract or sheep serum water. Cultures could not be obtained in the absence of both kidney tissue and ascitic fluid, which might each have contained living cells; modified media were inferior generally to a mixture of the above two components.

The results obtained by the use of the substituted media indicate that growth was derived primarily from the inoculum and not from the kidney tissue or ascitic fluid. The original observation supporting this view is that relatively greater success was obtained when fragments rather than emulsions of poliomyelitic tissue were used. For it has been found that bacteria may be introduced from the outside into cultures of brain tissue from herpes-virus encephalitis (2) and from poliomyelitis (3) during the process of grinding the tissue in the preparation of emulsions.

Characteristics of the Globoid Body.—The globoid bodies were originally described as minute, globular structures, measuring from 0.15 to 0.3 micron in diameter, and arranged in pairs, chains, and small masses, according to the nature of the medium. In fluid medium, pairs and chains predominated; within the tissues of poliomyelitic patients or infected monkeys, only pairs or small masses were discernible, never chains. The reaction to Gram's stain was variable, that is, the bodies retained the stain feebly in earlier generations, but more intensely in older cultures. In peptone medium the organisms appeared to be larger and retained the Gram stain more firmly.

In initial cultures, considerable difficulty was encountered in demonstrating the bodies during the first 4 or 5 days. They could be detected more readily on the sixth or seventh day, and maximum growth was obtained from about the eighth to the twelfth day. After 3 or 4 weeks, however, the bodies became enlarged and irregularly stained, although very minute structures, barely visible, were still present. These altered cultures were filterable through Berkefeld filters, just as were the globoid organisms.

It would appear, therefore, from the original description in which the authors stressed the ability of the globoid bodies to form definite colonies and to show a distinctive morphology, that the bodies were unequivocally organisms.

Pathogenicity in Monkeys.—The original paper emphasized the point that only exceptional cultures possessed pathogenicity in the monkey. Furthermore,

the limit beyond which the virus itself was considered not to be infective was that represented by a second transfer of the original virus in the culture medium.

Two sets of inoculation experiments were reported: one comprising fluid and solid cultures of globoid bodies derived from four human cases, and the other, cultures from the M.A. virus highly adapted by prolonged propagation in monkeys.

The mixed cultures of the human strains, in the third generation, were inoculated into a monkey, which developed characteristic experimental poliomyelitis. A second passage from this animal to two normal monkeys, and later, a third passage to a single *rhesus*, proved successful. From three of these four animals, cultures of the microorganisms were recovered.

The monkey strains of globoid bodies were successfully inoculated as follows: One monkey was injected with a mixed culture representing the third and fifth generations, and a second, third and fourth animal, with fifth to sixth generation cultures, all developed typical poliomyelitis. The globoid bodies were recovered in cultures made from the brains of all these animals. Furthermore, from the second and third *Macacus rhesus* successful passage was effected through two successive series of animals. In addition, a fifth monkey was inoculated with a mixed culture representing the eighteenth and twentieth generations. This animal died on the third day from a secondary infection but the inoculation of a filtrate of the site of inoculation in the brain into a normal *cynomolgus* was followed, after 13 days, by characteristic experimental poliomyelitis.

In one instance in the foregoing experiments, mention was made of the fact that characteristic experimental poliomyelitis was induced with a culture derived from an affected monkey which, in turn, had also been inoculated successfully with globoid bodies.

To sum up, the original report offered evidence of specific pathogenicity of globoid bodies even to the eighteenth or twentieth generation and of the successful recovery of a virulent culture from an animal injected with the globoid bodies themselves.

In a later article, Flexner, Noguchi, and Amoss (4) extended the studies on the globoid bodies and found that the microorganisms survived in the semisolid medium kept at room temperature or at 37°C. for over 13 months. A test for virulence showed these cultures to be pathogenic 6 months later, or 19 months after isolation. In this communication the authors also reported the preparation of "mass" culture medium and the employment of the prodigious number of globoid bodies so obtained for pathogenicity tests. The mass cultures were injected three or four times, since the earlier studies had revealed that only exceptional growths were active and that repeated injections of the ordinary virus (5) sometimes induced paralysis. Thus, one *rhesus* monkey was inoculated intraspinally four times at intervals of 6 to 11 days with mass cultures representing the fourth to thirteenth generations. 5 days after the last injection the monkey developed characteristic experimental poliomyelitis. Another monkey was injected intraperitoneally four times with the same material, and at the same intervals as in the preceding

experiment, and typical poliomyelitis was induced. The nervous tissue of these two monkeys, glycerolated for 6 and 70 days respectively, proved infectious. The effect of the repeated injections is cumulative, for it was shown by a control test that the final, or fourth injection, by itself, failed to affect a normal animal.

The experiments described led to the inference that there is a wide fluctuation in pathogenic action of the globoid bodies, and only exceptional strains are infective. In addition, it was emphasized that the recovery of the bodies in culture from affected monkeys is made only with difficulty. Finally, it was pointed out that, by means of mass cultures, the bacterial nature of the globoid bodies is unequivocally established.

The next communication, by Amoss (6), again presented evidence to indicate the fact that the globoid bodies are microorganisms, and that after long cultivation *in vitro* they become saprophytic and grow more readily in a considerable variety of media. Moreover, Amoss reported the recovery of two additional, but non-pathogenic, cultures from monkeys with experimental poliomyelitis induced by human and M.A. virus. He emphasized in this paper a fact that had already been pointed out in the previous articles (1, 4), namely, that highly parasitic cultures are refractory to artificial cultivation.

Smillie (7) contributed a series of twenty-two cultures of globoid bodies in fluid and semisolid media obtained from the tissues of seven monkeys with experimental poliomyelitis. Of these, eight different strains were inoculated into corresponding *rhesus* monkeys. Five animals were unaffected but three, receiving fourth and fifth generation growths, showed some degree of paralysis after intracranial or intraspinal injection. These latter reactions could not, however, be definitely proved as poliomyelitic. Smillie also found that established cultures can survive in the icebox for months; that dye indicators inhibit their growth; that the preferable pH of the medium is at blood neutrality; that strict anaerobiosis is essential and that body fluids, especially ascitic fluid, are required for their proper development. Most noteworthy is the fact, as shown also by Amoss (6), that after having once become established and accustomed to artificial media, the globoid bodies grow more readily and may be more easily transferred.

The observers mentioned in the preceding paragraphs have therefore defined the characteristics of a true culture of globoid bodies. In view of the minute size of the microorganisms, emphasis was laid on the possible confusion which could arise in distinguishing them from granular precipitates in the culture medium. Hence the criteria of a culture should comprise the following principles: (a) characteristic growth in special, anaerobic, fluid medium; (b) definite colony formation; (c) distinctive growth in mass cultures, and (d) agreement with the other properties already mentioned in the foregoing résumé. Finally, (e) in respect to pathogenicity, attention has been called by

the early investigators to the fact that only exceptional cultures show infectiousness and that the saprophytic strains are cultivable with difficulty while parasitic ones are even more refractory. On the other hand, definite experimental poliomyelitis has been induced in monkeys with the first, third, fourth, fifth, sixth, eighth, eighteenth, and twentieth generations of the globoid bodies. The eighteenth generation has been calculated to represent a dilution of $1:24^{17}$ of the original virus—considerably beyond the limits of its inherent infectivity. In one instance, a culture in semisolid medium, in its eighth generation and kept at 37°C . for 13 months, had not lost its infectiousness, whereas ordinary virus in a similar medium survives no longer than 20 to 30 days (4). Furthermore, the microorganism has been recultivated from the tissues of monkeys in which poliomyelitis was induced by the globoid bodies (in one series, from all of four cases) and such strains have, in turn, again produced the experimental disease.

With this definition of a culture of globoid bodies in mind, we can now compare the results of other investigators with those of Flexner and Noguchi (1, 4), of Amoss (6), and of Smillie (7).

In 1918, Heist, Solis-Cohen, and Kolmer (8) reported the isolation from human and monkey poliomyelitic material of four different strains of globoid bodies. These cultures agreed morphologically and culturally, in fluid, semisolid, and mass-culture medium, with the descriptions originally given (1, 6, 7). Furthermore, a definite distinction was made by Heist and Solis-Cohen (9, 10) between globoid bodies and ordinary streptococci—a finding also reported by Smillie (7) and by the prior investigators. Although the cultures of Heist and his co-workers were carried successfully through at least ten generations, no inoculation tests were made.

On the other hand, the results of the cultivation tests reported by Tsen (11) are not so clearcut. After "many trials" he succeeded in finding organisms which he believed to be similar to globoid bodies. But they could not be maintained for more than three generations, and hence were not inoculated into animals. Since only fluid medium was used, and the "organisms" therein disappeared rapidly, it is likely that Tsen may have confused granular precipitate with the globoid microorganisms.

Into this category of granulations may also be placed the so-called globoid body cultures obtained by Foster (12) from the nasopharyngeal secretions of common cold cases, and those of Bradford, Bashford, and Wilson (13). Olitsky and McCartney (14), in discussing the significance of Foster's cultures, pointed out how the error of confusing precipitates with minute organisms may arise and, further-

more, attempted to indicate the methods by which such misinterpretations may be eliminated. In the case of Bradford and his co-workers, their cultures of so-called "globoid bodies," obtained from patients with influenza, trench fever, war nephritis, and a number of other diseases, were later shown to consist of artefacts (Arkwright—15)—a view which the investigators themselves finally accepted.

In a study concerning the etiology of epidemic encephalitis lethargica, Loewe and Strauss (16), using the fluid and semisolid culture media already described (1), recovered what was believed to be a minute, globular organism from the brain, from nasopharyngeal mucous membrane tissue and washings, from spinal fluid, and from blood of cases of the affection. These investigators stated that their filterable organism resembled in morphology and cultural characteristics the globoid bodies, but that it possessed distinctly different pathogenic effects. They have not, however, reported the distinctive bacteria-like growth, as described by Amoss (6), in mass-culture medium. The fact, however, that colonies were obtained indicates the occurrence of bodies of microorganismal nature in their cultures, which, the authors believe, should be placed in the globoid body group.

EXPERIMENTAL

As indicated in the introductory paragraph, we undertook to repeat the earlier work on the cultivation of the globoid bodies and, especially, to secure cultures which possessed pathogenic properties.

For this purpose, we had access to abundant material taken from monkeys which had developed experimental poliomyelitis after the inoculation¹ of a highly active mixture of two virus strains—M.A. and K—(17, 18) which had been propagated for many years in monkeys. The animals, as soon as definitely paralyzed and before becoming moribund, were etherized and the brain and spinal cord were removed with sterile precautions. The tissues were generally free from secondary bacterial contamination, or bacteria derived, during exposure, from the air. Inoculation of media was immediately carried out, using fragments of brain or spinal cord, or a Berkefeld V filtrate of a 5 per cent physiological saline suspension of these tissues.

Cultivation

The culture medium was that of Noguchi, consisting of ascitic fluid and a fragment of sterile, fresh, rabbit kidney.

In the preparation of initial cultures, we attempted to repeat precisely the original method described by Flexner and Noguchi (1). The fragment of rabbit kidney

¹ All inoculations in the monkey were carried out under ether anesthesia.

was placed in a test tube measuring 1.5x20 cm. The inoculum, consisting of either a small fragment of brain or of spinal cord, or of 0.5 cc. of the filtrate of these tissues, was next added and then about 15 cc. of sterile ascitic fluid. Finally, about 2 cc. of sterile liquid albolene was added in such a way as to form a layer over the surface of the medium. One set of tubes of each series was placed in the Boëz anaerobic jar (19), and a duplicate set kept outside. The incubation was at 37°C.

A modified technique was also used: sufficient 1 per cent neutral cysteine hydrochloride solution was introduced into the ascitic fluid to give a final concentration of 1:2000. The cysteine medium was sealed with solid petrolatum instead of liquid albolene. We found, however, that this modification did not lead to an increase in the number of positive "cultures."

As a rule, about 50 tubes of medium were inoculated during each original attempt at cultivation: two-thirds of them were set up as first described, and one-third with the cysteine.

After 10 to 14 days, the tubes were removed from the thermostat and a small amount of the medium was withdrawn from the bottom of each tube with a sterile capillary pipette, for film preparations. These preparations were allowed to dry in the air and, after being fixed by heat, were stained for 3 minutes with well-ripened, alkaline, methylene blue.

All tubes found by microscopic examination to be contaminated with ordinary, familiar bacteria were discarded. On the other hand, each culture thought to contain material resembling globoid bodies was subplanted to six or eight tubes of Noguchi's fluid medium and upon two rabbit-blood, dextrose-agar plates. In the case of each subplant, one-half the number of tubes and plates was placed in an anaerobic jar and the other half kept outside. In addition numerous uninoculated control tubes of the medium, and inoculated control tubes of dextrose broth were included in each series of transfers.

No characteristic macroscopic changes were visible in the inoculated tubes at any time. The opalescence described by Flexner and Noguchi (1) as appearing around the kidney at the end of the fifth day was not detected but a distinct clouding, possibly due to autolysis, was noted at the end of 24 hours.

With regard to the formation of colonies, which other workers (Flexner and Noguchi—1; Flexner, Noguchi, and Amoss—4; Amoss—6; Smilie—7, and Heist, Solis-Cohen, and Kolmer—8) agree in emphasizing as characteristic of the globoid bodies, and as indicative of their microorganismal nature, we were unable to secure any evidence. This may perhaps have been due to the fact that we did not employ the semisolid medium of Noguchi. However, no colonies could be detected on the plates, incubated anaerobically, which were made from tubes showing suspected growth. Nor were we successful in obtaining growth in the special mass-culture medium (4), although no less than twelve attempts were made by this method.

In view of our having used different media in this part of the work, any comparison to be made between our results and those of the original investigators should be based on findings in fluid cultures

To recapitulate: Efforts at cultivations were made with fragments of the brain or cord or with Berkefeld V filtrates of the nervous tissues of seven monkeys inoculated with poliomyelitic virus and suffering from experimental poliomyelitis. With these materials, 315 tubes were inoculated, thirty-six of which showed minute morphological particles or "bodies" suggesting microorganisms. The subplanting from these "positive" tubes was disappointing, owing to the failure to obtain secondary growths in later subplants or to failure due to contamination with ordinary bacteria. Thus, while all seven monkeys yielded minute particles suggesting growths in initial cultures, the "growths" derived from three animals failed after the first subplant, from one after the third, from one after the fourth, from one after the eighth, and from the last monkey after the eleventh subplant.

Morphology

The microscopic appearances to be described relate wholly to the fluid subplants and it should be recognized that because of the presence of the autolyzing kidney fragment and the resulting granular precipitate, it is just such cultures that are most subject to misinterpretations. Indeed, the distinction in early subplants between minute microorganisms and precipitate is so indefinite that we were led to apply the method of cataphoresis in order to attempt possible differentiation. We already knew that the poliomyelitic virus in nervous tissue suspensions migrates to the anode (20). Applying the same experimental procedures (21) to washed "cultures" in twenty-three tubes derived from six different "strains" in five separate tests, no conclusive evidence could be obtained, by this method, of differentiation of "microorganisms" from precipitate. For whatever picture was detected at the anode could be seen also at the cathode. On the other hand, control experiments with *Bacterium pneumosintes* and *Bacterium coli* were clearcut; there was active migration of both to the anode.*

Notwithstanding these facts, we were able to pick out thirty-six from 315 tubes in which the distinctions were sufficiently wide to suggest the possibility of microorganisms being present. The main points of

* In carrying out the cataphoresis experiments, we were aided by the effective cooperation of Dr. D. C. Hoffman of The Rockefeller Institute.

such distinctions are as follows: In early subplants, the minute and "globoid bodies" were single, in pairs, or in short chains, the individual bodies being of a fairly uniform appearance. In older subplants, the bodies were more numerous and in part formed agglomerated masses. In remote subplants, they were somewhat larger. The bodies stained well with well-ripened alkaline methylene blue and, as a rule, did not retain Gram's stain. In stained specimens the "bodies" appeared to be raised above the background of amorphous material, cellular detritus, and indefinite particles and were not refractile. It should be mentioned that in practically all film preparations we observed well-preserved kidney tissue cells and leucocytes, the latter probably derived from the ascitic fluid.

Power to Infect Monkeys

We pass now to the most significant part of our study, namely, the power of the "cultures" to infect and induce experimental poliomyelitis in monkeys. Indeed, in one series of subplants, derived from the brain and cord of a monkey experimentally infected with the virus described in Experiment 1, we were successful in producing experimental poliomyelitis in monkeys with the materials taken from "cultures" in the seventh, eighth, ninth, and tenth transfers. It should be noted that the seventh transfer represents a dilution of the original material cultivated of about 1.5×10^{-12} , and the tenth, of about 1.3×10^{-18} . The history of the monkey yielding the active material is as follows:

Experiment 1.—*Macacus rhesus* inoculated intracerebrally on February 8, 1929, with 1 cc. of the suspension of anodic material obtained by cataphoresis of brain and cord tissues from a monkey in the paralytic stage of the experimental disease (20). February 19, excitement, tremor, right facial paralysis, and ptosis were noted. February 20, ataxia, tremors, weakness of right arm and shoulder and both legs were observed. February 21, the animal was prostrate and unable to move its arms and legs. Etherized. The gross and histopathological examinations revealed characteristic lesions of experimental poliomyelitis.

Sixty-nine tubes were inoculated with the infected nervous tissue of this animal.

Of the sixty-nine tubes, eight showed what appeared to be a minute, "globoid microorganism." These were subplanted to sixteen tubes of which, after 12 days' incubation, five were "positive." A third subplant into thirty tubes yielded sixteen; a fourth into forty-two tubes yielded nine; a fifth of forty-five, eleven; a sixth of sixty-six, twenty; a seventh of sixty, thirty; an eighth of forty-eight,

thirty-seven; a ninth of thirty-two, four; and a tenth of thirty-two, five "positives." All the forty tubes of the eleventh subplant were contaminated by moulds and diphtheroid organisms. By the time the sixth subplant was reached, all the "positive" tubes had a common origin from one tube of the first transfer.

The medium is notably irregular and uncertain in its composition because of the variations in quality of the ascitic fluid and possibly of the kidney tissue. Noguchi (22) originally pointed out that certain samples of ascitic fluid were more suitable than others for cultivating spirochetes and other investigators have emphasized this factor as influencing cultures of globoid bodies (4, 6, 7). That uncontrolled fluctuating factors played a part in our results is indicated by the discrepancies arising in the several subplants. The irregularity in positive tubes is correlated with the number of "bodies" visible under the microscope: In the first three subplants they were few, from the fourth to the eighth subplant, many; with the seventh subplant they predominated, but in the ninth to eleventh they became very few and difficult to find.

Monkeys were inoculated with materials from the seventh, eighth, ninth, and tenth subplants of these "cultures." The material inoculated consisted of centrifuged sediment of the tubes, washed, as a rule, five times with physiological salt solution. In one instance (Experiment 2) to the washed sediment of the seventh subplant was added the unwashed sediment of the sixth and fourth subplants.

Experiment 2.—*Macacus rhesus* A. April 18, 1929, pooled material from the bottom of the positive tube in the sixth transfer of the culture obtained in Experiment 1 (hereafter designated as Bodies 1) was prepared and of this mixture 2 cc. were injected intracerebrally, 10 cc. were injected into the peritoneal cavity and 2 cc. into the spinal canal.

April 23, pooled material from the bottom of the positive tube in the fourth transfer of Bodies 1 was prepared, and of this mixture 2 cc. were injected intracerebrally, 9 cc. intraperitoneally, and 2 cc. intraspinally.

May 1, the material from the bottom of the positive tube of the seventh transfer was centrifuged at high speed for 15 minutes; the sediment was suspended in 3 cc. of physiological saline solution, and then centrifuged again. This was repeated three times. 1.75 cc. of the final suspension were injected into the left cerebral hemisphere.

May 11, tremor; right facial paralysis; ataxia; complete paralysis of left arm, and legs very weak.

May 12, prostrate. Etherized.

Autopsy.—Meninges normal. A very small area of softening was visible in the border of the right internal capsule, involving the lenticular nucleus. This site contained old blood and yellow pigment. The midbrain showed spotty translucent areas about 1 to 2 mm. in diameter. The cervical cord contained pink, translucent, soft areas in the region of the anterior horn cells.

Microscopical Examination.—Forebrain: A moderate mononuclear cellular reaction was present. Lateral to and partly involving the lenticular nucleus was a small area of softening containing red blood cells in various stages of degeneration. Around this area were many phagocytic microglia. Certain of the nerve cells showed varying degrees of degeneration and some of them were phagocytized.

Medulla: A slight mononuclear infiltration of the pia-arachnoid membrane was seen. Moderate perivascular infiltration was present. A certain amount of nerve cell degeneration with and without accompanying cellular reaction occurred.

Cord: A slight lymphocytic reaction was observed in the pia-arachnoid membrane, together with a marked perivascular mononuclear infiltration. In the cervical region there was almost complete destruction of both the anterior and posterior horn cells, with accompanying phagocytosis of the debris by microglia. The same reaction was present but was progressively less marked in the lower levels of the cord.

A 5 per cent saline Berkefeld N filtrate was prepared from portions of the cord and of this 1 cc. was injected intracerebrally into a monkey. This monkey developed typical experimental poliomyelitis within 6 days.

Cultivation experiments in Smith-Noguchi medium were carried out with fresh central nervous tissue from Monkey A. Seventy-one tubes were inoculated and after 14 days' incubation, five tubes contained bodies resembling those with which Monkey A had been inoculated.

Experiment 3.—*Macacus rhesus* B. May 1, 1929, 2.5 cc. of a saline suspension of washed materials from the bottom of the positive tube of the seventh transfer of Bodies 1 were injected into the left cerebral hemisphere.

May 6, slight tremor; ataxia; marked left facial paralysis; both arms practically completely paralyzed.

May 7, prostrate; facial paralysis increased. Etherized.

Autopsy.—At a site of inoculation there was a soft area 2 x 2 mm. containing a small amount of golden yellow pigment. No cyst formation or frank hemorrhage was observed. The region of the anterior horn cells in the spinal cord showed injection and slight hemorrhage. This was most marked in the cervical region.

Microscopical Examination.—Brain: Slight perivascular lymphocytic reaction in the region of the thalamus. Irregular nerve cell degeneration. The choroid

plexus was infiltrated with lymphocytes. In the sub-arachnoid space was a diffuse lymphocytic and mononuclear cellular reaction.

Medulla: Foci of mononuclear cells, some surrounding degenerated nerve cells, were present in the region of the fourth ventricle. A marked perivascular mononuclear infiltration occurred throughout the medulla. All stages of nerve cell degeneration were seen.

Cervical cord: A moderate meningeal and perivascular reaction was observed. The neurons showed marked destruction and active neuronophagia.

Thoracic cord: The meninges were thickly infiltrated with lymphocytes. The cells of the anterior and posterior horns showed marked acidophilic degeneration, but their outline was preserved. The nerve cells were surrounded by a large number of polymorphonuclear and mononuclear leucocytes. The same changes were observed in the lumbar cord.

Intervertebral ganglia: There was a marked interstitial lymphocytic reaction and a moderate acidophilic degeneration of the nerve cells.

Five monkeys were inoculated with suspensions and filtrates prepared from the cord of Monkey B. Three of these control monkeys developed typical experimental poliomyelitis within 7 days; the remaining two were unaffected.

Cultivation experiments in Smith-Noguchi medium were made with fresh material from the brain and spinal cord of Animal B. In the second transfer bodies closely resembling those inoculated were isolated.

Experiment 4.—*Macacus rhesus* C. May 15, 1929, 1.5 cc. of a saline suspension of washed material from the bottom of the positive tubes of the eighth transfer of Bodies 1 were inoculated into the left cerebral hemisphere and 1.5 cc. of the same suspension were injected into the spinal canal.

May 21, tremors; slight ptosis of both upper eyelids; ataxia; partial paralysis of both arms and shoulders; legs very weak. Etherized.

Autopsy.—Meninges normal. Site of inoculation used for touch preparations. A definite small hemorrhage was seen in the striate body. The cervical cord revealed definite areas of congestion and hemorrhage in the region of the anterior horn cells. These changes were less marked in the lumbar and sacral cord.

Microscopical Examination.—Site of inoculation: No bodies were seen in touch preparations. Lateral to the anterior part of the striate body was a narrow zone of softening containing red blood cells, granular debris, and a moderate amount of pigment. About the periphery of this area was a large number of glial nuclei.

Cord: The anterior horn cells showed varying degrees of degeneration, some being slightly involved while others were completely destroyed. A diffuse cellular infiltration of polymorphonuclear and mononuclear leucocytes was present. This reaction became less marked in the lumbar and sacral regions.

Intervertebral ganglia: A diffuse interstitial lymphocytic infiltration was present and a moderate number of nerve cells showed partial or complete degeneration.

A 5 per cent saline Berkefeld N filtrate was prepared from the brain and cord of Monkey C. 1 cc. of this filtrate was injected into the left cerebral hemisphere of a normal control monkey. Within 8 days the animal developed typical experimental poliomyelitis.

Cultivation experiments in Smith-Noguchi medium were made with fresh tissue of the central nervous system from Monkey C. After 14 days' incubation, one of forty-seven inoculated tubes contained bodies similar to those with which the monkey had been inoculated.

Experiment 5.—*Macacus rhesus D.* May 31, 1929, 1.25 cc. of a washed saline suspension of the material from the bottom of the positive tubes of the ninth transfer of Bodies 1 were injected into the right and left cerebral hemispheres.

June 6, tremor; ataxia; both arms paralyzed; legs weak.

June 7, prostrate. Etherized.

Autopsy.—The meninges were slightly injected. The left site of inoculation showed an old hemorrhage with some yellow pigment and an area of softening extending into the internal capsule and the caudate nucleus. A similar picture was present in the right site of inoculation. Typical translucent, congested and hemorrhagic areas were visible in the medulla and in the region of the anterior horn in the cervical cord. No organisms were seen in preparations made from the sites of inoculation.

Microscopical Examination.—The site of inoculation showed a hemorrhage with hemosiderin pigment and was surrounded by phagocytic mononuclear cells. A slight diffuse increase in glial nuclei was present. Nearly all of the anterior horn cells in the cervical region had disappeared. Many foci of phagocytic mononuclear cells were present. A moderate perivascular lymphocytic reaction was observed.

A 5 per cent saline suspension was made of the cord of Monkey D. 0.5 cc. was inoculated into the left cerebral hemisphere of a normal, control monkey, which in the course of 7 days developed typical experimental poliomyelitis.

Cultivation experiments in Smith-Noguchi medium were made with the fresh central nervous tissue from Monkey D. None of the forty-eight tubes showed bodies similar to those with which Monkey D had been inoculated.

Experiment 6.—*Macacus rhesus E.* May 31, 1929, 1.25 cc. of a washed saline suspension of the material from four negative tubes of the ninth transfer of Bodies

1 were inoculated into the right and left cerebral hemispheres. This material contained no demonstrable bodies.

The monkey remained well and was discarded after 1 month's observation.

Experiment 7.—*Macacus rhesus* F. June 14, 1929, 1.5 cc. of washed saline suspension of material from the bottom of five positive tubes of the tenth transfer of Bodies 1 were injected into the right and left cerebral hemispheres.

June 20, excited; tremors; right deltoid weak.

June 21, prostrate. Etherized.

Autopsy.—There was slight meningeal congestion. An area on the left side, greenish gray to red in color, was filled with tenacious, soft, elastic material which contained staphylococci. The area extended from the superior surface of the frontal lobe to a region near the internal capsule, 1 to 2 mm. wide, and spread out at the base to a pyramidal area about 4 mm. in diameter. On the right side a similar condition prevailed, although the area was not as large but more hemorrhagic and extended almost to the internal capsule. The fourth ventricle revealed the usual picture of poliomyelitis, but its edges were bulging and the glistening surface showed pink, turgid, tiny, translucent, somewhat hemorrhagic areas. The cervical cord exhibited the characteristic injection and translucence of the anterior horns, but in the dorsal and lumbar cord the pinkish tinge was not as prominent.

Microscopical Examination.—Forebrain: At the site of inoculation an elongated area was seen extending from near the cortex to the external surface of the lenticular nucleus. This area contained erythrocytes, polymorphonuclear and endothelial cells, and granular debris. A small amount of golden yellow pigment was present. The tissue forming the border of this area comprised a mass of phagocytic mononuclear cells and proliferated neuroglia; there was also perivascular infiltration by lymphocytes. Lymphocytes infiltrated the pia-arachnoid. In the medulla there were present occasional nerve cell degeneration and neuronophagia with a moderate perivascular infiltration.

Cord: Cervical region. Moderate lymphocytic infiltration of the meninges. Almost all neurons showed acidophilic degeneration and some were in the process of neuronophagia. The gray matter was diffusely infiltrated with lymphocytes and with occasional polymorphonuclear cells.

Thoracic region: Here the cord was the seat of an intense reaction. No normal appearing nerve cells were present, all being degenerated and fragmented, the subject of active neuronophagia. Very marked extra-adventitial infiltration was noted.

Lumbar region: Lesions of meningeal mononuclear infiltration; nerve cell degeneration; neuronophagia; perivascular infiltration with some diffuse lymphocytic and mononuclear cellular infiltration throughout the gray matter. In general, the reaction was less marked here than in the cervical or thoracic cord.

Intervertebral ganglia: Moderate diffuse lymphocytic and mononuclear cell infiltration; a few necrotic nerve cells in various stages of degeneration with some showing neuronophagia.

A 5 per cent saline suspension was made of the cord of Monkey F; 0.5 cc. was inoculated into the left cerebral hemisphere of a normal monkey, which developed typical experimental poliomyelitis after an incubation period of 7 days.

Cultivation experiments in Smith-Noguchi medium were made with fresh tissue of the central nervous system of Monkey F. Two of twenty-eight tubes showed bodies similar to those employed in the inoculation of Monkey F.

Experimental 8.—*Macacus rhesus* G. June 14, 1929, 1.5 cc. of a washed saline suspension of the material from five negative tubes, that is, free from "globoid bodies," of the tenth subplant of Bodies 1 were inoculated into the right and left cerebral hemispheres. The monkey remained well throughout the period of observation.

One observes from the preceding experiments that the inoculation in monkeys of a saline suspension of the washed sediment from Smith-Noguchi cultures of poliomyelitic tissue induced, in the seventh, eighth, ninth, and tenth transfers, the clinical syndrome and the pathological effects characteristic of experimental poliomyelitis. It is noteworthy that in the successful experiments, the washed material contained small bodies resembling the globoid bodies described by Flexner and Noguchi. Equally important is the fact that in parallel experiments with material derived from the ninth and tenth transfers, the washed sediment of tubes showing similar bodies was active in monkeys while that of tubes not containing the bodies was inactive.

SUMMARY AND DISCUSSION

The study here reported concerns attempts at bacteriological cultivations with fragments of brain or cord, or with Berkefeld V filtrates of the nervous tissues, from seven monkeys successfully inoculated with poliomyelitic virus. With these materials, 315 tubes were inoculated, of which thirty-six showed minute bodies resembling the globoid bodies described by Flexner and Noguchi. However, a study of subplants from these minute, morphological particles did not convince us that we had in hand actual cultures of the globoid bodies, or indeed of any living microorganism.

Nevertheless, when washed sediments from subplants of one of the

strains, representing the seventh, eighth, ninth, and tenth transfers, were inoculated into monkeys; the clinical signs and pathological effects characteristic of experimental poliomyelitis could be induced. The virulence of the "cultures" could not be ascribed to carrying over the original material into these remote subplants since the seventh transfer represented a dilution of the original cultivated material to about 1.5×10^{-12} , and the tenth, to about 1.3×10^{-18} if one assume, as the transfer technic justifies, a thorough mixing of the contents of each tube. On the contrary, it appears as if the poliomyelitic virus had multiplied *in vitro*, and had increased as a consequence of being in a medium of a modified living tissue-cell culture. For in practically all specimens we observed many well-preserved kidney tissue cells and leucocytes, the latter probably derived from human ascitic fluid, a component of the Smith-Noguchi medium. In this connection, it should be mentioned that the several lots of ascitic fluid used in the cultivation tests were recently obtained from patients and employed from a week to a month after their collection.

There remains for consideration the problem of the selective pathogenicity of the "cultures:" only the material of those tubes of the ninth and tenth transfers which showed the "globoid bodies" proved pathogenic; those respective tubes of the same transfers which were free from the minute bodies but apparently identical in all other respects, were avirulent. It may be that the virus was adsorbed to the particular bodies which we have found in the "cultures" and which resemble closely the globoid bodies of Flexner and Noguchi. Further elaboration of this study would be necessary, however, before such an inference could be regarded as a definite hypothesis.

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A METHOD FOR SUPRAVITAL STAINING OF ANIMALS WITH NEUTRAL RED AND ITS PRESERVATION IN PARAFFIN SECTIONS

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The only method which satisfactorily preserves neutral red in sections of tissue supravitaly stained is that reported by Cash (1)—or certain modifications of it—for the study of monocytes and epithelioid cells in the lungs of animals experimentally infected with tuberculosis.

Cash slowly injected into the ear vein 15 cc. of a 1 per cent solution of neutral red, dissolved in normal salt solution. In this manner he secured a true supravital staining of the connective tissue cells of the lungs. Any damage to the cells brings about diffuse staining of cytoplasm and nuclei and obscures those structures which are significant in the so-called supravital reaction. It is an essential part of the technique, therefore, to secure this reaction first. 10 minutes after the injection the rabbit was quickly killed. Thin slices of the lungs were fixed in Zenker-formol solution for from 12 to 24 hours. These blocks of tissue were quickly dehydrated, without washing, in acetone, cleared in benzene or xylol, and rapidly embedded in paraffin. Thin sections were then stained with various dyes, avoiding alcohols and water as much as possible. This method gave uniformly good results with lung tissue.

Prior to the experiments of Cash, McJunkin (2) reported a method for staining tissue supravitaly and embedding it in paraffin. He exsanguinated animals from the carotids under light ether anesthesia and then injected saline solution, saturated with neutral red, directly into the tissues. The tissues were fixed in a Zenker formol solution. McJunkin states that the contact with the dye is irregular and that no observations can be made within the areas in which the cells are injured. Indeed, the weakness of the interstitial method of introducing the neutral red is the unevenness and uncertainty of obtaining a supravital reaction of the cells.

Gardner (3) states that the technique of McJunkin proved to be inapplicable

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for use on lung tissue and he therefore modified the methods of McJunkin and Cash. He injected the dye solution either intratracheally or intravenously. The fixation was carried out in the same fluid as that used by McJunkin, except that the formaldehyde was carefully adjusted to a pH of 7.6 with sodium hydroxide. Gardner claims that the sodium has a specific mordanting effect upon staining.

It is well-known that the supravital technique of staining monocytes in the blood or tissues is the most reliable method we have for the accurate differentiation of these cells from other cytological elements. Good descriptions of it can be found in the papers of Simpson (4) and Sabin (5). Forkner (6) has recently described in some detail the methods, as used in Dr. Sabin's laboratory, of applying the technique to tissue cells. This description includes some minor modifications in the methods as originally reported by the above authors. Forkner (7) has shown that lymph nodes from certain areas of the body contain an abundance of monocytes, but that the exact relations of these cells in the tissues could not, by the methods then available, be demonstrated. The technique here recorded was elaborated for the study of this point. It has been found to be equally applicable for the study of all supravital stainable cells throughout the body. The process of staining and rapid fixation was developed by the writer. The method of dehydration and embedding is a modification of that recorded by Cash (1), to whom I am indebted for suggestions in the development of this technique.

Method

Apparatus.—An instrument which has been of great value in this work is a modified, self-retaining cannula. It is made by inserting a fish-hook (Fig. 1, *a*), the curve of which has been eliminated by heating and straightening (Fig. 1, *b*), into the lumen of a No. 18 or 20 gauge transfusion needle in such manner that the barb of the hook protrudes over the edge of the needle, as shown in the accompanying figures (Fig. 1, *c*). About 1.5 or 2 cm. of the shaft of the hook lies within the lumen of the needle and if a slight bend is made in this shaft, it will retain its position within the lumen but can be removed for cleaning. When such a needle is inserted into the heart, or into a large vessel, the barb catches and prevents its coming out. In this way the labor, time, and trauma caused by the insertion and ligation of a cannula are avoided.

A large pressure flask (about 5 liters) is tightly stoppered and connected by means of rubber tubing with three outlets (Fig. 2) to (1) a blood pressure pump, (2) an Erlenmeyer flask containing 500 to 1000 cc. of 0.6 per cent neutral red (Grübler,

vital nach Ehrlich) in 0.9 per cent sodium chloride solution, and (3) Erlenmeyer flask containing 500 cc. of Zenker's fixing fluid containing 15 per cent formalin and no acetic acid (Fig. 2). The flasks containing neutral red solution and the fixing fluid each has a glass tube extending to its bottom, in order that all the fluid can be pumped out when an increased pressure in the flask obtains. These glass tubes

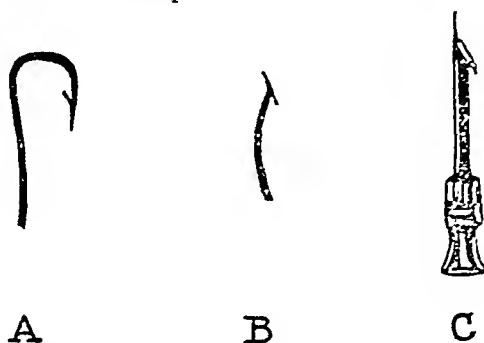


FIG. 1. Diagram illustrating construction of special cannula for perfusion of small animals through the heart.

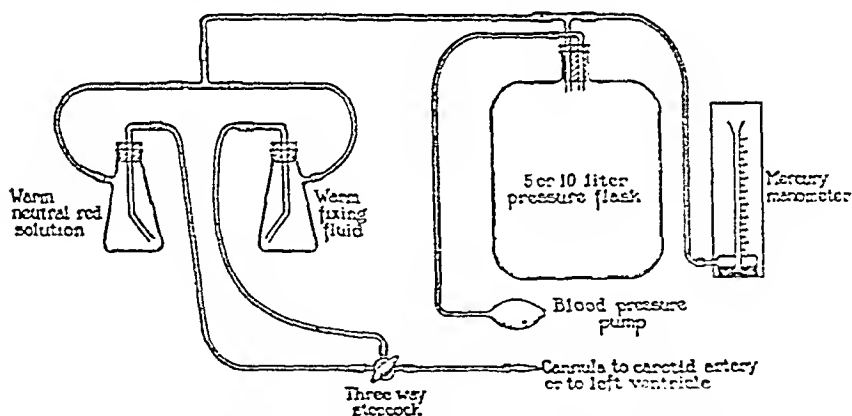


FIG. 2. Apparatus used for staining of tissues and fixation of supravital dyes in cells of the entire body.

are connected by about 1.5 meters of small rubber tubing to two of the outlets of a three way stopcock. The remaining outlet of the stopcock is connected by a short rubber tube to the barbed cannula, as above described.

Staining of Tissues with Neutral Red.—An animal (rabbit, rat, guinea pig) is anesthetized with ether and fastened, ventral side up, with cords to an animal board. A jugular vein is exposed and ligated at the end proximal to the heart. An ordinary cannula is inserted into the distal segment. The animal should now

be under deep anesthesia. Two routes may be used for injection of the neutral red. One can either insert a cannula into a common carotid artery, ligating the distal segment of the artery, and inject the neutral red through the carotid artery into the aorta, or a window about 2 cm. square can be quickly made in the lower left chest near the sternum, care being exercised not to injure the lungs. With a pair of blunt forceps, the parietal pericardium is torn away, the apex of the heart held, and the special self-retaining cannula quickly inserted into the left ventricle and the neutral red solution allowed to pass into the heart, under a pressure of from 100 to 150 mm. of mercury. It is understood, of course, that the injection system must be free of air. At the moment of injection, the cannula in the jugular vein is opened and the blood and neutral red solution are allowed to escape. The cords binding the legs are immediately released to promote a ready circulation through the entire vascular system. The perfusion proceeds for from 15 to 25 minutes, at the end of which time the entire animal is deep red in color. The perfusion fluid should be warmed to from 38° to 40°C. and the rubber tubing carrying the fluid immersed in a vessel of warm water at this temperature. If the heart becomes greatly distended, an incompetency of the mitral valve results with a back flow of neutral red into the lungs through the pulmonary veins and considerable fluid may thus escape through the trachea into the mouth. This may be avoided by clamping the trachea with a hemostat or by clamping off the left auricle. Likewise, intercostal or internal mammary arteries may be clamped off if excessive fluid is being lost.

The amounts of fluid described above are for rabbits. For animals of smaller or larger size, the amounts can be more or less proportionate. Considerably less neutral red solution is required if one injects by way of the carotid artery instead of through the left ventricle. In the rabbit or larger animals, the injection through the carotid is the method of choice. With rats, mice, and all small animals, the route through the left ventricle is much more feasible and it is for these smaller animals that the special barbed cannula is especially adapted.

Fixation of Neutral Red in Tissues.—After all of the neutral red has been passed through the circulation, the stopcock is adjusted so that the fixing fluid warmed to body temperature is injected immediately following the neutral red and through the same channels. About 500 cc. of fixing fluid perfused over a period of from 10 to 20 minutes will reach every organ. The cannulae and all instruments are then immersed in running water to prevent tarnishing with mercury. Instruments made of nickel-steel are not as readily injured by the fixing fluid. Organs are now removed and small blocks placed in a vessel or vessels containing the fixing fluid, where they are allowed to remain for from 12 to 24 hours at room temperature.

Dehydration and Embedding.—It is necessary that blocks be cut not over 2 mm. in thickness, in order that rapid dehydration can take place. Such blocks are dipped in tap water and blotted to remove the excess of fixing fluid. They are then carried through the following solutions, blotting gently after each step up to No. 7.

Solution 1.	Absolute ethyl alcohol—rinse for 1 minute	
" 2.	" " "	5 minutes
" 3.	Pure acetone	20 "
" 4.	" "	20 "
" 5.	" "	20 "
" 6.	Xylol	20 "
" 7.	"	20 "
" 8.	56°C. Paraffin	1-2 hours
" 9.	56°C. "	1-2 "

The blocks are then embedded in 56°C. paraffin.

Staining.—The sections are cut from 4 to 10 microns in thickness, mounted on slides previously prepared with albumin fixative, and allowed to dry in a 37° incubator for 4 hours, or preferably over night. The sections on the slides can be excellently treated by the drop bottle method as follows: (1) Xylol to remove paraffin 10 to 20 seconds; (2) Absolute alcohol 5 seconds; (3) 95 per cent alcohol 3 seconds; (4) 95 per cent alcohol containing 1 per cent iodine 15 seconds; (5) 95 per cent alcohol several washings; (6) Absolute alcohol 5 seconds; (7) Clear in xylol; (8) Mount in balsam.

With the above method the macrophages, monocytes, and other vitally stained cells will stand out beautifully with the identical appearance of these cells, as seen in supravitaly stained films of living cells.

An excellent counterstain can be employed after step (5) as follows:

(6 a) Distilled water for just long enough to overcome surface tension (3 to 5 seconds); (7 a) Goodpasture's acid polychrome methylene blue, full strength, for from 2 to 7 seconds; (8 a) Rinse quickly with water; (9 a) 95 per cent alcohol 5 seconds; (10 a) Absolute alcohol 5 seconds; (11 a) Xylol to clear; (12 a) Mount in balsam. Other methylene blue solutions may be used, but Goodpasture's permits of rapid staining, does not obscure the neutral red, and causes no troublesome precipitates.

Another method which is probably better than any other for demonstrating and preserving the cytoplasmic structures in supravitaly stained macrophages and monocytes, and at the same time giving a satisfactory nuclear stain and a beautiful staining of the reticulum-fibers is a modification of Foot and Mènard's (8) method for staining reticulum by silver impregnation. The method is carried out in exactly the same manner as described by Foot, except that the sections are not bleached. When stained in this manner, the neutral red is of course washed out or obscured, but all the cytoplasmic structures which are stained with neutral red become intensely black. The

monocytes, pre-monocytes, and macrophages show the typical characteristics, except that the cytoplasmic bodies are black. Detailed descriptions of these cells stained in the tissues by the above methods can be found in an accompanying paper (9).

SUMMARY

A simple, rapid method for staining all the supravitally stainable cells in the body, as in supravital preparations with neutral red, is described, together with a method for faithful preservation of the dye in paraffin sections. The essential points of the technique are, first, to secure the reaction of cells to neutral red which corresponds to the so-called supravital technique, involving the reaction of only those substances which respond to the dye while the cell is living; and second, to preserve the stain through the processes of fixation, embedding, and counterstaining.

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THE ORIGIN OF MONOCYTES IN CERTAIN LYMPH NODES AND THEIR GENETIC RELATION TO OTHER CON- NECTIVE TISSUE CELLS

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PLATES 12 TO 15

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The purpose of this study was to determine the position of monocytes in certain lymph nodes and, if possible, to ascertain from what cells they arise. The writer (1) has shown in a previous communication that lymph nodes from different parts of the body exhibit marked differences in cytology and that developing monocytes may be found as normal constituents of all the lymph nodes of the rabbit, except the large mass lying in the mesentery of the intestine. At the time of the earlier communication, no reliable method had been found for demonstrating the relationship of monocytes within lymph nodes to other tissue elements. The method used in these observations, which has been fully described in another communication (2), gives true supravital staining of the cells, faithfully preserves this reaction in paraffin sections, and shows their relation to other cellular elements.

The origin of the monocyte has been a much debated question. Various theories to the effect that monocytes arise from myeloblasts, lymphocytes, endothelium, histiocytes, reticulo-endothelial cells, and from undifferentiated cells have been advanced. Each is supported by a certain amount of evidence. However, no clear cut experiments are recorded to show that under normal conditions any of these cells give rise to monocytes. The writer agrees with Maximow (3) that the development of monocytes in the blood-forming tissues—"with the exception perhaps of the red pulp of the spleen"—has never been demonstrated.

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Description of Cells

The protocols to follow will state briefly what kinds of cells have been encountered in various tissues and will describe their morphological relations to each other. It seems expedient, therefore, to describe in some detail the cytological characteristics of the types of cells under discussion with special reference to supravital staining with neutral red and Janus green.

Primitive Undifferentiated Cells (Fig. 10, "c").—These elements can also be called mesenchyme cells. They are undifferentiated and possess multiple potentialities for the formation of various mesenchymal tissues. Entirely unstained by the supravital method, they are pale cells, with a round or oval nucleus and moderate cytoplasm containing no vitally stained granules. They have been described and illustrated by Cunningham, Sabin and Doan (4), by Forkner (1, 5), and others. They may be found in any of the blood-forming organs and probably possess multiple potentialities. In lymph nodes they doubtless represent the stem cells from which lymphocytes, monocytes, macrophages, and fibroblasts are derived.

Lymphocytes.—Maximow (6) believes that lymphocytes are not definitive cells, but are the stem cells from which various other cells develop. In the opinion of others, they represent a definitive cell. They appear as small, intermediate, and large forms which take very little or no neutral red into their cytoplasm when stained supravitally. Their nuclei are usually round, but may be oval, indented, or even horseshoe shaped. The chromatin is characteristically in heavy splotches. These cells usually have in their clear, hyalin cytoplasm a few round, globular, refractive, neutral red bodies. These bodies may at times be numerous and may even be arranged in the bay of the nucleus, in a rosette fashion about the centrosphere. However, the character of the neutral red granules, the character of the mitochondria, the type of motility, and the other general characteristics of the cells do not permit of their confusion with monocytes. Lymphocytes possess abundant mitochondria in their cytoplasm. These structures in themselves are of a different morphology than those in monocytes. They are larger and coarser. The mitochondria are apt to be in clumps near the nucleus, but at times are found scattered through the cytoplasm. The lymphocyte possesses quite a characteristic type of motility. It moves with the nucleus in the forward portion of the cell, as has been shown by Sabin (7).

Monocytes. (Figs. 1, 2, 4, 5, 6, 10).—Other terms which have been applied to these cells are: large mononuclears and transitionals of Ehrlich, endothelial leucocytes, and blood histiocytes. When stained with neutral red and Janus green, the monocytes of the rabbit possess features which permit of their easy differentiation from all other elements in the blood or tissues. They are large in size, as a rule, often exceeding all of the other blood cells in this respect. The

with Giemsa stains. Their cytoplasm is very lightly stained and presents a very pale appearance

Monoblast (Fig. 10).—This term has been frequently used in the literature to designate the precursor of the monocyte. Bloom (10), however, in a recent paper, has made the statement that monoblasts do not exist. The demonstration in lymph nodes of monocytes in all stages of development allows one to shift the term monoblast from a more or less theoretical name to its proper place as a term designating that particular cell which is derived from a primitive undifferentiated cell and which is the precursor of the pre-monocyte. It is identified in preparations from peripheral lymph nodes by the fact that it is found in close association with pre-monocytes and monocytes in clumps of these cells. It appears to be identical with the pre-monocyte, except that it possesses no demonstrable segregation apparatus, that is, no supravitality stained neutral red bodies. It is easily demonstrable in paraffin sections of supravitality stained lymph nodes (Fig. 10).

Mesenchymal Macrophages.—There is no group of cells which has received more names, nor about which there is more confusing terminology than the cellular elements included under this term. The various terms present in the literature to designate the phagocytic cells under discussion are: macrophage, clasmatocyte, adventitial cell, resting wandering cell, rhagiocrine cell, reticular cell, reticulo-endothelial cell, endothelial leucocyte, pyroll cell, and histiocyte. The writer (11) has recently critically reviewed the literature on this subject and has proposed that we revert to the original descriptive term "macrophage" which was first applied to these cells by Metchnikoff (12, 13). It was proposed to limit the term, conforming to our present knowledge, and to include only those cells of mesenchymal origin which possess the property of phagocytosis of débris, or of staining intensely with moderate doses of vital dye in the living state. Where particular groups of macrophages are to be discussed in this paper, they will be designated as: macrophages of the lymphoid tissue, macrophages of the adventitia, macrophages of the common connective tissue, macrophages lining the lymph sinuses, etc. Such cells, supravitality or vitally stained, have been abundantly illustrated in the literature by many investigators (14, 15, 8, 16, 1). They are cells of large size (Figs. 4, 9). The nucleus is usually near the center of the cell, is round or oval, and has a delicate chromatin network. The cytoplasm is abundant and possesses few or many irregularly sized and shaped bodies, scattered throughout without any pattern or definite arrangement. These bodies may represent débris which the cell has phagocytized, or in many instances, they represent vacuoles in which the neutral red or vital dyes have been assembled. The cytoplasmic structures are not only heterogenous in size and shape, but also frequently show various shades of staining. This is in contrast to the uniform homogeneous appearance of the cytoplasmic structures in monocytes. The macrophages lining the sinuses (reticulo-endothelial cells—Fig. 8) are similar in their reactions to dyes and foreign material. The latter cells are fixed, are often flattened or elongated, and can be selectively stained with vital dyes. By appropriate experiments, reported in the protocols of this

paper, it is shown that these cells are among the first to stain with vital trypan blue, whereas the phagocytic macrophages of the reticular syncytium and the free macrophages in the lymphoid tissue may be almost entirely unstained with few doses of this dye. However, this difference is probably dependent on the fact that the former, by virtue of their anatomical position, are more directly exposed to the material passing through the lymph node. It is also significant that the macrophages lining the lymph sinuses (reticulo-endothelial cells) and the phagocytic macrophages of the reticular syncytium are identified with the formation of reticulum fibers, whereas free macrophages are not.

Plasma Cells.—These cells should be described here because they frequently are found in lymph nodes of rabbits and other mammals. So far as the writer is aware, there has been only one description of these cells as they appear in supravital preparations. An analysis of the literature on this point will be considered in the discussion at the end of this paper. On the whole, they are about the size of intermediate lymphocytes, but may be smaller and are often somewhat larger. They possess, as a rule, more cytoplasm in relation to the nucleus than do lymphocytes. The nucleus is generally round or slightly oval, and is often eccentrically placed in a manner which is well-known for these cells. The one special characteristic possessed by these cells, when seen in supravital preparations stained with neutral red and Janus green, is that the cytoplasm has a peculiar and characteristic appearance. It is homogeneous and possesses a faint yellow tinge, almost like that of a nucleated red cell in which the full quota of hemoglobin is not present. Because of this fact, plasma cells in lymph nodes have often been mistaken for developing erythrocytes. Plasma cells in lymph nodes often contain in their cytoplasm, as do lymphocytes, one or a few small, refractive, globular, neutral red bodies. Mitochondria are frequently numerous and of the same type as those described for lymphocytes. In no instance have plasma cells been seen possessing segregation granules resembling those found in monocytes or pre-monocytes. They are easily distinguished from these latter cells.

Materials and Methods

Normal rabbits, guinea pigs, and rats vitally stained with trypan blue have been used in these experiments. The method has been completely described in an accompanying paper (2) which should be consulted for repetition of these experiments. Let it suffice to say here that sections of tissues which have been stained supravitaly alone or in combination with vital staining are in many instances more instructive than the living supravitaly stained cells. The chief advantage of the method is that the supravital staining is preserved in paraffin sections. A few typical protocols of experiments are given in the following pages.

PROTOCOLS OF EXPERIMENTS

Experiment 1.—Rabbit 2. The animal fresh from stock was etherized. Neutral red, 0.6 per cent, in 0.9 per cent sodium chloride solution, dissolved by warming and then filtered, was injected at body temperature and at 150 mm. of mercury pressure, through a cannula into the exposed left ventricle. A cannula was placed in the right jugular vein, through which the perfusion fluid and blood were allowed to escape. The animal was thus perfused with about 800 cc. of the neutral red solution over a period of 25 minutes. Immediately following, Zenker-formol solution was similarly injected at body temperature through the same cannula, over a period of 20 minutes. Tissue from the various organs was removed, placed in fixing fluid of the same kind, and allowed to remain over night. The method of staining, fixation, dehydration, embedding and counterstaining is described in detail in the accompanying paper (2).

The organs were all well stained except the bone marrow, which contained only a few areas of stained cells in the immediate neighborhood of the nutrient arteries. Much neutral red had accumulated in the stomach and intestines which were dilated and the contents of which were dark red in color.

Peripheral lymph nodes were normal in size and stained a dark red color. On histological examination, all the nodes were essentially alike, except that some contained more monocytes than others. Some of the lymph nodes were heavily laden with monocytes which were situated in the lymphoid tissue in and around the primary follicles. Also in some nodes monocytes were present in large numbers in certain of the secondary follicles, the so-called germinal centers. Most of the germinal centers, however, contained very few or no monocytes. The monocytes, as seen in sections, were often in clumps of from four to twenty or more cells (Figs. 1, 3, 5, 6, 10). These clumps were characteristically a part of the parenchyma of the lymphoid tissue and were not free or separated from the lymphoid cells by any perceptible boundary. Many monocytes were also found diffusely scattered through the cortical tissue, but were almost never present in the medullary cords. These cells were easily recognized by their characteristic appearance. It is interesting to note that they were not in any way related, in their position, to cells lining the sinuses. It is true that occasional monocytes are found in the sinuses (Fig. 5) and in the medullary cords, but this is not at all a common occurrence. Their histological appearance cannot be confused with that of the macrophages lining the lymph sinuses (Fig. 8), the cells of the so-called reticulo-endothelium. Monocytes in various stages of development can be easily demonstrated, particularly in areas where monocytic cells are numerous (Fig. 10). The life cycle can be traced from undifferentiated cells through the stages of monoblasts and pre-monocytes to mature monocytes.

Free macrophages, so-called reticular cells, are likewise numerous in the peripheral lymph nodes, but are not as abundant as monocytes. They are found within the sinuses, in the parenchyma of the lymphoid tissue, and may be scattered through the follicles (Figs. 4, 9), being present at times in the secondary follicles.

These free macrophages vary tremendously in size from that of a large lymphocyte to giant cells with abundant cytoplasm and several nuclei. As a rule the cytoplasmic bodies are scattered throughout the cell without pattern and conform closely to the description previously given. However, certain unmistakable, free macrophages have the cytoplasmic bodies arranged in the form of a very large rosette. This rosette is not composed of the uniform, fine, brick red, non-refractive, neutral red bodies typical of the monocyte, but, on the contrary, the vacuoles are coarse, irregular, of different shades of color, and possess different refractive properties. By diligent search, cells can be found which cannot with certainty be placed in one or the other group. These cells must be considered carefully, for they suggest that under physiological conditions monocytes may be transformed into macrophages. This point will be considered in the discussion to follow.

Plasma cells were found in the lymph nodes of this animal (Rabbit 2). In some areas they were numerous. These areas were chiefly located in the less dense lymphoid tissue and not in the follicles themselves. The plasma cells were easily distinguished from all the other cellular elements.

Mesenteric lymph nodes differ considerably in their cytology from the other lymph nodes, as discussed in detail in an earlier paper (1). The essential differences are: (a) almost complete absence of monocytes in mesenteric lymph nodes, but an abundance in peripheral lymph nodes; (b) presence of more macrophages, chiefly those lining the sinuses, than in peripheral nodes; (c) presence of many more large lymphocytes with abundant basophilic cytoplasm in the mesenteric than in the peripheral lymph nodes.

Spleen.—No developing monocytes were found. Occasional, fully developed monocytes were present. Many free macrophages were present, some of which contained fragmented cellular débris.

Thymus.—An abundance of free macrophages was present, chiefly limited to the cortical zone. No monocytes could be found in either the cortical or medullary zone.

Liver.—No monocytes were present. Many macrophages (Kupffer cells) were in the capillaries. The organ presented an entirely normal appearance.

Omentum.—Paraffin sections demonstrated a few small groups of monocytes, and this possibly represents a place of development for these cells. Their number, however, was insignificant when compared with the number found in the lymph nodes other than the mesenteric mass of lymphoid tissue. Many monocytes appeared to lie on the surface of the serosa, as though they had been present in the peritoneal fluid and had adhered to the omentum. It must be stressed, however, that in supravital films of the omenta of normal rabbits, rats, and guinea pigs, milk spots can be seen composed almost exclusively of monocytes, others containing primarily macrophages, others lymphocytes, and still others primitive undifferentiated cells. Such findings indicate that in the omentum there are present many cells possessing mesenchymal potencies. Many free macrophages were found in the omentum of the animal described in this protocol. Also, many elongated,

fixed macrophages in close association with fibroblasts and connective tissue fibers could be demonstrated.

Bone marrow.—The bone marrow was not well stained in this animal but will be described in some of the protocols which follow.

Many other organs were studied, but their description is not relevant.

Experiment 16.—Rabbit 16. The animal was used in an attempt to inject neutral red more directly into the vessels of the posterior extremities in order to stain supravivally the bone marrow cells. The abdomen was opened under ether anesthesia and a cannula inserted into the abdominal aorta, just above the pelvic brim. Neutral red, 500 cc. of a 0.6 per cent solution in normal saline, was then slowly perfused through the vascular system of the posterior extremities under the same conditions as those in Experiment 1. The same fixing fluid was also used.

After this process the bone marrow was removed intact with blunt forceps. Dehydrating, clearing, and embedding were carried out as in Experiment 1. The marrow was distinctly red, particularly in the neighborhood of the nutrient vessels. Lymph nodes from the popliteal and inguinal groups were also saved.

Microscopically the marrow showed large areas which were ideally stained with neutral red in a manner which renders the differentiation of the cellular types easy. The bone marrow from the long bones of rabbits is very active in blood formation and contains granulocytes, erythrocytes, and megakaryocytes in all stages of development. Macroscopically it is red and highly vascular. It is not within the scope of this paper to describe the histological details of the findings, except to say that monocytes cannot be found in any part of the marrow. Free macrophages are scattered sparingly in the tissue. Macrophages lining the capillaries in the marrow, the so-called reticulo-endothelial cells, are abundant and for the most part are elongated and contain coarse, neutral red bodies in their cytoplasm. They resemble the macrophages lining the lymph sinus in lymph nodes. In no instances can cells be found which can be interpreted as transitional phases between macrophages and monocytes.

It had been demonstrated in the above experiments that all supravivally stainable cells in the body could be easily stained with neutral red and the color preserved in paraffin section. Vital staining with dyes such as trypan blue, Niagara blue, and lithium carmine has long been employed to mark out the cells of the so-called reticulo-endothelial system. It was proposed, therefore, at the suggestion of Professor Aschoff, to use the combined methods of supravital staining with neutral red and vital staining with trypan blue to determine whether or not the developing monocytes really belong to the reticulo-endothelial system, the system of histiocytes, or of mesenchymal macrophages. Paraffin sections of such tissues show that the cytoplasmic structures in the cells which have been previously vitally stained with trypan blue and subsequently counterstained with neutral red present a violet color, whereas those cells which have not been capable of taking up trypan blue and contain only neutral red possess a pure red color, just as though no trypan blue had been introduced. The following experiment was performed.

Experiment 17.—Rabbit 17. A normal rabbit was given a series of five intravenous injections of a sterile, 1 per cent, aqueous solution of trypan blue. The injections were given every second day. 24 hours after the last injection of trypan blue, the animal was perfused, through the left heart, with 1000 cc. of 0.5 per cent neutral red, dissolved in normal salt solution. The perfusion and fixation were carried out under the same conditions as in Experiment 1. The general appearance of the tissues changed from a blue to a purple color during the course of the perfusion with neutral red.

Microscopic examination of the lymph nodes demonstrates clearly that the pre-monocytes and monocytes in the lymph nodes are not stained with trypan blue. On the other hand, many free macrophages (reticular cells), many phagocytic cells of the reticular syncytium, and practically all of the macrophages lining the lymph sinuses (reticulo-endothelial cells) contain an abundance of trypan blue, as well as neutral red. It must be stated that many macrophages in the lymphoid follicles contain very little or no trypan blue. Also, certain cells can be seen to be more abundant than in normal animals, which morphologically represent stages between typical monocytes and typical macrophages and which are, almost without doubt, transition phases in the development of the latter from the former. These cells are likewise intermediate in their reaction to trypan blue, taking only a small amount of this dye. All transition phases can be demonstrated between monocytes containing neutral red but no trypan blue, and macrophages containing an abundance of both colors.

Another experiment was undertaken to confirm these findings. It was proposed to inject much more of the trypan blue to determine whether excessive doses of this dye would alter the above findings.

Experiment 18.—Rabbit 18. This animal was treated with five intravenous injections of 6 cc. each of a 1 per cent aqueous solution of trypan blue, followed by a series of four injections of 8 cc. each of the same solution. These injections were also given on every second day. 24 hours after the last injection, the animal was perfused with 800 cc. of 0.7 per cent neutral red solution and then with fixing fluid, as in the previous experiments.

The results of this experiment are, so far as the point under discussion is concerned, in entire agreement with those of Experiment 17. There are, however, a few additional points which should be mentioned. It will be remembered that in Experiment 17 the macrophages lining the lymph sinuses were strikingly colored with the trypan blue, whereas only a portion of the free macrophages and macrophages of the reticular syncytium were stained. In Experiment 18, practically all of these macrophages contained an abundance of trypan blue,

whereas monocytes lying in close proximity to these cells remained unstained with vital dye, but were richly colored with neutral red (Fig. 9). This fact demonstrates that, even with prolonged administration of large doses of vital dye, the monocytes and pre-monocytes of the lymph nodes remain unstained. In this experiment there were more cells which might be interpreted as intermediate stages from monocytes to macrophages (Fig. 9).

It is interesting also in this experiment that the macrophages of the liver capillaries (reticulo-endothelial or Kupffer cells) were increased considerably in number and for the most part contained a large amount of trypan blue. Also in the sinuses, one sees occasional typical monocytes, often three or more in a clump. Moreover, typical transition forms from monocytes to macrophages can be demonstrated. This finding in the liver will be discussed in another portion of this paper.

DISCUSSION

The literature on the origin of the monocyte shows that no one has as yet proved the derivation of this cell from any fixed cell in the body. Numerous investigations have been reported on the mode of production of these cells under pathologic or tissue culture conditions. These reports are contradictory, excellent workers stating that they arise from endothelium, lymphocytes, myeloblasts, histiocytes, etc., and equally competent investigators denying these statements. The report contained in the present contribution deals with normal animals and demonstrates a site of origin and development of monocytes under physiological conditions. It shows that monocytes are formed in certain specific blood-forming organs and in this respect are analogous to all the other structural elements of the blood. The analogy can be carried even further for, like other white blood cells, they are shown to be derived from primitive, undifferentiated, cellular elements and their various stages of development can be clearly demonstrated.

It is not denied that monocytes may, under abnormal conditions, be abnormally produced in abnormal locations. On the contrary, it has been shown conclusively by numerous investigators that under experimental or pathologic conditions, monocytes may arise in various organs and tissues of the body. This, however, is likewise true of lymphocytes and granulocytes.

The methods here reported for the study of the development of

monocytes in lymph nodes have been equally applicable to the study of these and related cells in all organs of the body. They have demonstrated conclusively that, in the bone marrow of the long bones of rabbits, developing monocytes are not to be found, although the marrow is very active for the production of granulocytes and erythrocytes. This confirms the observations of Sabin and Doan (17). These authors gave the first direct, conclusive evidence that in the rabbit, under physiological conditions, monocytes are not derived from myeloblasts or any other cell in the bone marrow.

Monocytes have been said to arise in considerable numbers in the spleen. Sections of supravitality stained spleens of the animals used in these experiments have failed to show more than occasional monocytes and very rarely pre-monocytes. It is thus apparent that the spleen plays no significant part as an organ in which monocytes are produced.

Maximow (3), Bloom (10), Masugi (18), Rhoads and Parker (19), Witts and Webb (20), and others have maintained that monocytes do not occur in any significant numbers in lymph nodes. All of these investigators employed the supravital technique, together with other methods, for their studies. Maximow (3) and Bloom (10) have used these results as one of their reasons for affirming the original contention of Maximow that monocytes are formed from lymphocytes in the blood stream by individual transformation of the latter cells. On the other hand, Masugi (18) made use of the same evidence to uphold his view and that of Aschoff (21) that monocytes are formed by transformation from histiocytes and reticulo-endothelial cells. It has been possible to utilize the same evidence for the support of each view because a gap has been present in our knowledge of the development of monocytes.

Although Bloom (10) recently has reported that all transition stages between lymphocytes and monocytes may be found in the blood of animals experimentally infected with *B. monocytogenes*, this has not been confirmed by Witts and Webb (20). Furthermore, no cells are shown in Bloom's figures, or described in his paper, which correspond to the early developmental forms of monocytes, as seen in peripheral lymph nodes. It is not intended to imply that the development of monocytes from lymphocytes in the blood stream is impossible, but to indicate that the evidence is insufficient to prove that under physiological conditions this is the mechanism for their formation.

In other experiments of Bloom (22, 23) he cultured lymph from the thoracic duct and after a time recovered monocytes. But one cannot be certain that all the cells in the thoracic duct are lymphocytes. It is not uncommon to find undifferentiated cells which possibly possess multiple potentialities. Even if one assumed that a pure culture of lymphocytes could give rise to a pure culture of monocytes, one would still have to show that this is the mechanism by which monocytes are produced under physiological conditions.

None of my evidence supports the view that monocytes are, under normal conditions, derived from ordinary blood vessel endothelium, macrophages lining the lymph sinuses (reticulo-endothelium), or from free macrophages. Undoubtedly however, desquamated reticulo-endothelial cells do occur under certain conditions in the blood stream, as pointed out by Mallory (24), and many others. Sabin and Doan (25) maintain that under normal conditions there is a practically constant desquamation of endothelial cells into the circulating blood in rabbits and man. It is difficult to conceive of how these cells could give rise to monocytes. Furthermore, no direct evidence for such transformation is recorded in the literature. The reports of Schilling (26) and of others that monocytes have been seen to arise from the Kupffer cells in the liver demand discussion, since it has been shown in this communication (Experiment 18) that monocytes may be found in the liver capillaries in small numbers when animals are vitally stained with trypan blue. In addition, transition forms between monocytes and macrophages and between monocytes and epithelioid cells are demonstrable. It is probably such findings in pathological material that have been responsible for the theory that monocytes arise from Kupffer cells. The evidence, as will be pointed out presently, is decidedly against the theory of the transformation of Kupffer cells into monocytes.

On the other hand, one does find evidence for the hypothesis that monocytes may be transformed into free macrophages, and this probably represents one of the modes of formation of the latter cells. Such proof is recorded in the protocols of this paper. It is consistent with Simpson's (8) production of macrophage showers in the circulating blood. These showers were generally preceded by a marked monocytosis and the presence of cells which probably represented transition phases between the two types of cells. A typical protocol shows that the shower phenomenon first occurred after twenty-six injections over a period of 2 months. Masugi (18) has recently contested the findings of Simpson (8), although he seems not to have repeated the experiments. He explains the macrophage showers of Simpson as due to aspirated pericardial fluid.

Tissue cultures of the buffy coat of the blood (27, 28) have likewise shown that monocytes readily may be converted into macrophages. Carrel and Ebeling (29) compared the results of tissue cultures of monocytes from the blood and macrophages from the subcutaneous tissues of chickens. They found a transformation of monocytes into macrophages.

From the experiments recorded in this communication and from the experiments of many workers in the past, it appears to be proved that macrophages in lymph nodes may arise directly from the undifferentiated cells of the reticular syncytium, or that they may arise from monocytes which, in their turn, are derived from the same mother cells.

A schematic outline of the development of monocytes and macrophages in lymph nodes is presented in the accompanying diagram.

Diagram Representing Normal Development of Monocytes and Macrophages in Lymph Nodes

Common Endothelial Cell

- (a) Lines ordinary blood vessels.
- (b) Does not produce reticulum.
- (c) Does not stain with vital or supravital dyes.
- (d) Does not phagocytize debris, inhibitant.
- (e) May degenerate into blood as transient

Primitive Cell (Undifferentiated mesenchyme cell)
(Undifferentiated, non-phagocytic cell of reticular syncytium)
(a) May produce reticulum fibers.
(b) Contains no supravital or vital stain-able granules.
(c) Possesses multiple potentialities.

Monoblast

- (a) May or may not be identified with production of reticulum fibers.
- (b) Somewhat more differentiated than primitive cell.
- (c) Found in association with the monocytes.
- (d) Contains no vitally or supravital stain-able granules.
- (e) Not found in blood.

Pre-Monocyte

- (a) Does not form reticulum fibers.
- (b) Somewhat more differentiated than monoblast.
- (c) Found in association with monocytes.
- (d) Contains the beginning of a rosette of neutral red bodies.
- (e) Does not stain with vital dyes.
- (f) Purely found in normal blood.
- (g) May be experimentally produced in blood.

Monocyte

- (a) A fully differentiated cell.
- (b) Contains many neutral red bodies, usually in form of a rosette.
- (c) Is smaller than pre-monocyte.
- (d) Is a normal constituent of the blood.

Fired Macrophage

- (Phagocytic cell of reticular syncytium)
- (a) Produces reticulum fibers.
 - (b) Stains with supravital and vital dyes.
 - (c) Phagocytic for debris.
 - (d) Cytoplasmic structures have no characteristic pattern.

Macrophage Lining Sinus

(Reticulo endothelial cell)

- (a) Produces reticulum fibers.
- (b) Stains with vital and supravital dyes.
- (c) Phagocytic for debris.
- (d) Cytoplasmic structures have no characteristic pattern, size, or shape.
- (e) May become detached to form free macrophage.

Free Macrophage

- (a) Does not produce reticulum fibers.
- (b) Actively phagocytic for debris.
- (c) Stains with vital and supravital dyes.
- (d) No pattern to cytoplasmic structures.
- (e) Is a wandering cell.
- (f) Occurs in blood as transient inhibitant.

All the cells mentioned in the diagram may be seen in peripheral lymph nodes when stained and sectioned according to the writer's methods (2). They may originate in other manners besides those there given, but the diagram indicates one common method of their origin under physiological conditions. The peripheral lymph nodes are the only organs of rabbits in which one can constantly find monocytes in all stages of development.

McJunkin (30) has thought that monocytes are derived from what he calls "reticular lymph vessel endothelium" in lymph nodes. His methods, in my hands, have proved unsuccessful for the demonstration of monocytes, supravitality stained in paraffin sections of lymph nodes. On the other hand, many free macrophages and macrophages lining the lymph sinuses can be stained and demonstrated by his methods. Possibly McJunkin has confused these elements with monocytes.

What is the relation of monocytes to the reticulo-endothelial system? The experiments (Nos. 17 and 18) recorded in the protocols have demonstrated that monocytes and pre-monocytes, in the lymph nodes, remain unstained when the animals are repeatedly injected over a long period of time with trypan blue. These facts are in agreement with the general opinion that monocytes do not stain with vital dyes. There is one exception, however, which must be mentioned here. Doan, Sabin, and Forkner (31) have shown that some monocytes, epithelioid cells, and epithelioid giant cells may be stained vitally when directly exposed to trypan blue over a long period of time. This result might have been predicted, since Kiyono (14), Evans and Scott (15), and others, have demonstrated that in connective tissues even fibrocytes may become vitally stained after prolonged treatment with trypan blue or Niagara blue. The only conclusion which one safely can make with regard to the relation of monocytes to the reticulo-endothelial system is that they do not stain vitally by the usual methods which are used to demonstrate this system.

The question of the relationships between lymphocytes, monocytes and plasma cells in the rat has been discussed by Bloom (32). He has concluded that plasma cells often possess rosettes of neutral red bodies and that all transitions exist between plasma cells and monocytes in lymph nodes. The present work on rabbits does not support the conclusions of Bloom.

The question naturally arises: How do monocytes reach the blood stream, a point which has already been discussed by the writer (1). They probably gain admission to the capillaries by means of their own motility in much the same manner as do the granular leucocytes. It is true that in the peripheral lymph nodes developing monocytes are frequently found in close proximity to the capillary vessels (Fig. 10). It is also probable that many lymphocytes which develop in the spleen and lymph nodes wander into the blood stream.

SUMMARY

1. The theories for the origin of monocytes from myeloblasts, lymphocytes, endothelium, macrophages, and primitive cells are reviewed and considered.

2. Monocytes in all stages of development have been demonstrated to be present constantly in large numbers in all the lymph nodes of the body, except in the large mesenteric group.

3. The relations of these cells to undifferentiated cells, lymphocytes, macrophages, plasma cells, and endothelium are described.

4. The origin of adult monocytes from primitive undifferentiated cells through the stages of monoblasts and pre-monocytes is described and illustrated.

5. The demonstration in certain lymph nodes of innumerable monocytes in all stages of development permits of a shifting of the term "monoblast" from a more or less theoretical name to its proper place as a term designating that particular cell which is derived from a primitive undifferentiated cell and which is the immediate precursor of the pre-monocyte.

6. The term "pre-monocyte" is proposed to designate the intermediate stage between the monoblast and the mature monocyte.

7. Evidence is advanced to show that monocytes are an independent strain of cells, but that under physiological conditions they may be transformed into macrophages, this representing at least one way in which the latter cells normally are produced.

8. In no organs or tissues other than in certain specific lymph nodes, chiefly the peripheral group, can one constantly find monocytes in all stages of development.

9. Developing monocytes occasionally may be found in small num-

bers in the spleen, mesenteric lymph nodes, Peyer's patches, subcutaneous connective tissues, lungs, and omenta of normal rabbits, but their presence is by no means constant and their numbers are insignificant in comparison with those found in the peripheral lymph nodes.

10. Monocytes and pre-monocytes do not stain by the common methods used for the demonstration of the reticulo-endothelial system and therefore must be considered for the present as independent of this system, except in so far as monocytes may be transformed into macrophages.

11. Plasma cells, stained with the supravital technique, as seen in lymph nodes, are described. No basis has been found for the theory that plasma cells and monocytes are closely related structural elements.

I wish to express my appreciation and gratitude to Professor Ludwig Aschoff, in whose laboratory this work was done, for his many helpful suggestions.

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EXPLANATION OF PLATES

PLATE 12

FIG. 1. Peripheral lymph node of a normal rabbit. $\times 1000$. Paraffin section. Stained supravitaly with neutral red. Here is an area of developing monocytes in the less dense lymphoid tissue at the periphery of a primary follicle. This is not an isolated or single group of monocytes. The picture may be duplicated many times in almost any section of the peripheral nodes, when properly stained. All stages are present, from monoblasts to mature monocytes with well-formed rosettes.

FIG. 2. Peripheral lymph node of a rabbit. $\times 820$. Paraffin section. Stained supravitaly with neutral red. Counterstained lightly with Goodpasture's acid polychrome methylene blue. This figure shows developing monocytes within a primary follicle of the lymph node. The size of the cells can be compared with that of the lymphocytes at the left of the figure. The delicate, faint outlines of the nuclei can be clearly seen.

PLATE 13

FIG. 3. A follicle in a peripheral lymph node of a normal rabbit; a low power view of the same area shown in Fig. 2. $\times 289$. Paraffin section. Supravitaly

stained with neutral red. Lightly counterstained with Goodpasture's acid polychrome methylene blue. Not all follicles contain monocytes. Many contain more monocytes than this and some may be almost entirely composed of such cells. It will be seen that they are independent of reticulo-endothelium and of common blood vessel endothelium. They develop from the undifferentiated parenchymal cells of the stroma.

FIG. 4. Peripheral lymph node of normal rabbit. $\times 1200$. Paraffin section. Stained supravitaly with neutral red and counterstained by a modification of Foot and Menard's rapid silver method. The upper part of the photograph represents the peripheral portion of a primary follicle. In the center, at the periphery of the follicle, two monocytes with well-developed rosettes can be seen, "a." To the right is a free macrophage showing the character of the cytoplasmic structures in these cells, "b."

FIG. 5. Peripheral lymph node of a normal rabbit. $\times 1200$. Paraffin section. Staining as in Fig. 4. Here monocytes and pre-monocytes are shown in a peripheral sinus. They rarely appear in the sinuses. They are shown here as free cells which have probably made their way into the sinus by means of their own motility.

FIG. 6. A group of monocytes, pre-monocytes, and monoblasts in a peripheral lymph node of a normal rabbit. $\times 1200$. Staining as in Fig. 4.

PLATE 14

FIG. 7. Peripheral lymph node of a normal rabbit. $\times 1200$. Stained as in Fig. 4. Here is shown a group of early pre-monocytes and monoblasts. The former have small rosettes not fully developed, whereas the latter are entirely similar, except for the absence of the neutral red bodies.

FIG. 8. An area of lymphatic tissue between follicles of lymphoid tissue of a normal rabbit. $\times 1200$. Stained as in Fig. 4. The macrophages of the sinuses (reticulo-endothelial cells) are shown. These cells contain reticulum fibers and also irregular masses of neutral red. Monocytes cannot be found developing from these cells. Compare the monocytes of Fig. 7 with the reticulo-endothelial cells of Fig. 8.

PLATE 15

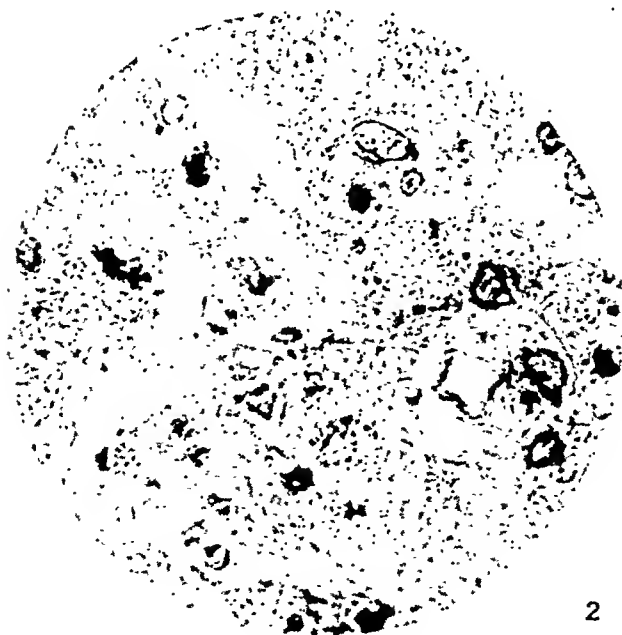
FIG. 9. Peripheral lymph node of rabbit treated with 9 doses of trypan blue (Experiment 18). Subsequently stained supravitaly with neutral red. Paraffin section $\times 820$. Here can be demonstrated intermediate stages in the transformation of monocytes into free macrophages. "a" points toward a cell which has a large rosette of neutral red bodies. In addition, a few globules of trypan blue have been taken into the cell. The granules of the rosette are larger than those usually seen in monocytes. It is a transition form but still possesses most of the characteristics of a monocyte. The cell to the right of the arrow leading from small letter "a" represents another step in the transition. It possesses an abundance of both neutral red and trypan blue. The cell indicated by "b" is a genuine macrophage which has probably developed from a primitive cell directly without first having

been a monocyte or pre-monocyte. It contains both dyes in abundance. "c" indicates a fully developed monocyte. It contains much neutral red, but no trypan blue. "d" indicates a developing monocyte, a pre-monocyte, with a characteristic rosette containing only neutral red bodies.

FIG. 10. An area of developing monocytes closely adjacent to a capillary blood vessel in a peripheral lymph node. Paraffin section $\times 820$. Stained as in Fig. 1. The small letters "a" point to cells which are regarded as monoblasts. They do not possess rosettes and yet are somewhat more differentiated than the primitive cells "c." The letter "b" indicates a pre-monocyte and "d" a well-developed monocyte. At the upper right hand corner a small capillary blood vessel is seen.

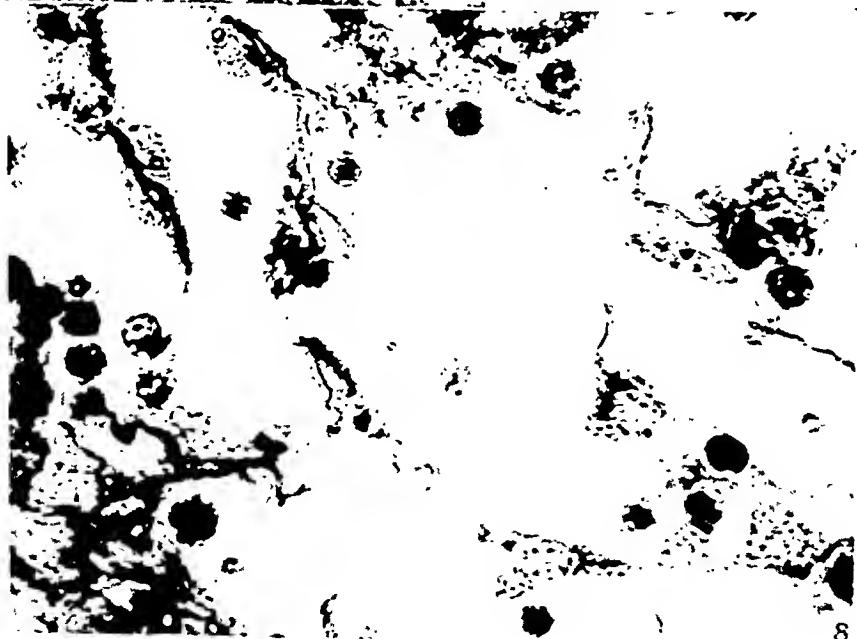
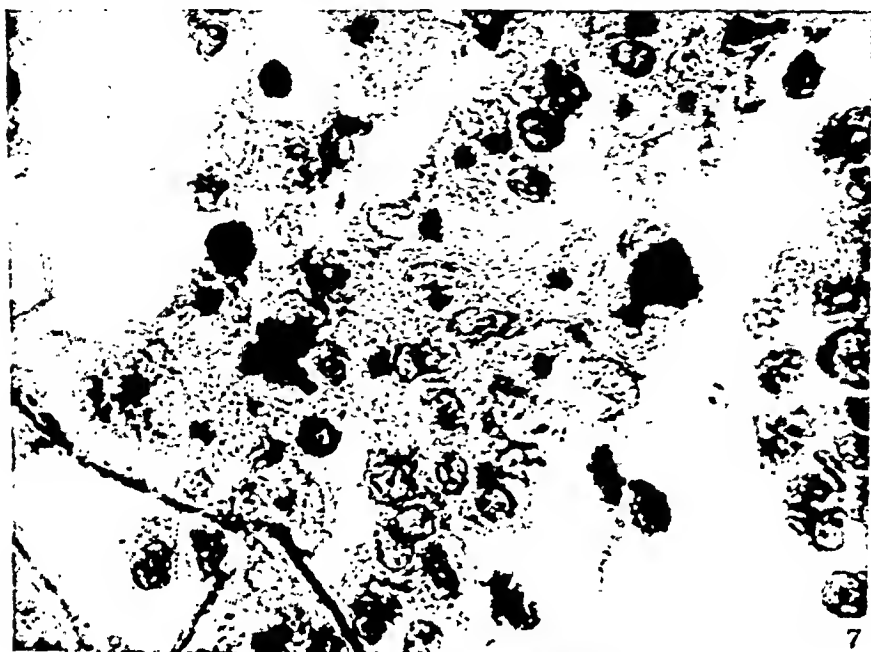


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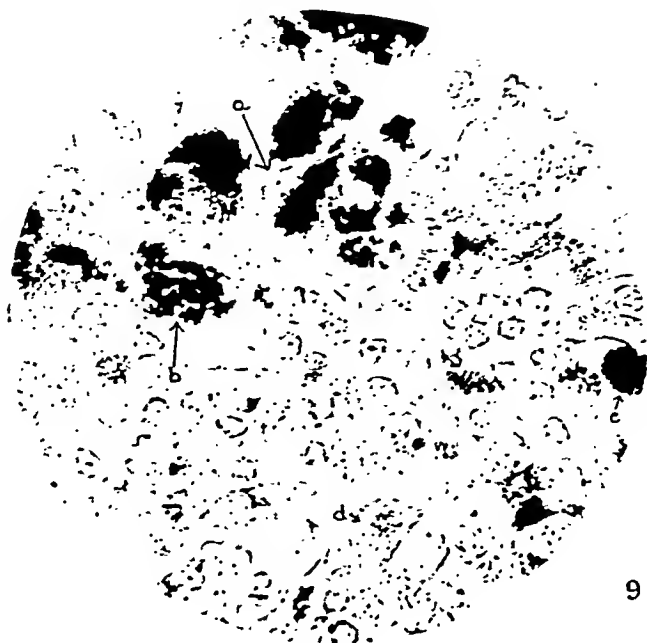


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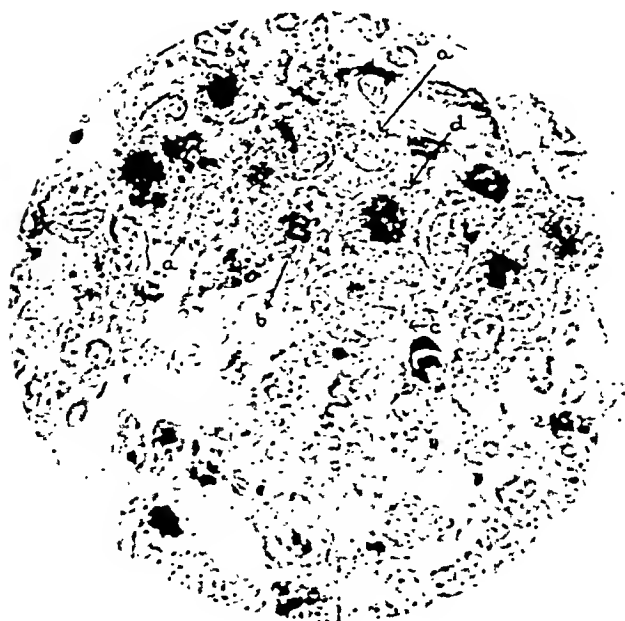




Under the first hypothesis, $\beta_1 = 0$, $\beta_2 = 1$, $\beta_3 = 0$.



9



10

THE SUSCEPTIBILITY OF MARMOSETS TO YELLOW FEVER VIRUS

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PLATE 16

(Received for publication, May 16, 1930)

Previous studies at this laboratory have demonstrated a relative susceptibility to yellow fever virus in the Capuchin (1, 2), "spider," "woolly," and "squirrel" (3) monkeys of Brazil. In the present paper it is proposed to discuss transmission experiments with certain more primitive genera, the members of which are usually called "marmosets" in English and "saguins" in Portuguese. These are very small and rather delicate monkeys, not particularly suitable for laboratory experimentation. It has been our experience that the mortality is rather high among recently captured specimens, but that the survivors which become accustomed to captivity can be used after a few weeks with less likelihood of deaths from unexplained causes during the course of the experiments.

Stokes, Bauer, and Hudson (4) inoculated marmosets with yellow fever virus in Africa. Apparently, the lesions in their Marmoset 63 were similar to those found by us in *C. albicollis*. They say in regard to their experiment: "Although there was no obvious other cause of death, the lesions in this animal did not furnish sufficient evidence for the conclusion that it had died of yellow fever infection."

Genus *Callithrix*. True Marmosets. *C. albicollis* (Spix)

Callithrix albicollis is found in great numbers in the environs of Bahia. Twenty of this species were used in the experiments here reported.

It was found comparatively easy to pass yellow fever virus through these marmosets and back to *rhesus* monkeys, either by blood transfers

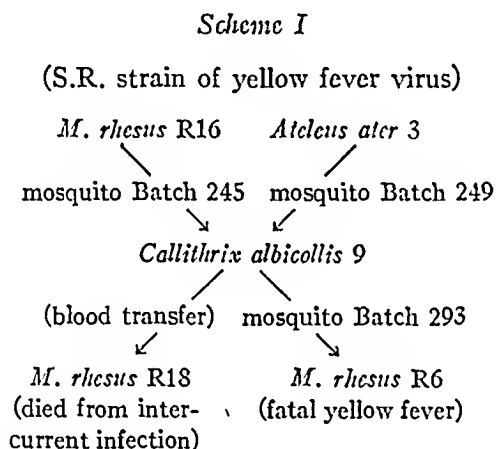
Temperature Records of Monkeys Infected with Yellow Fever

Genus and number	Date of beginning of experiment	Virus strain	Manner of infection	Transfer from:	Temperature A.M. and P.M. (degrees F.)										Outcome	
					Initial day of experiment	1	2	3	4	5	6	7	8	9		10
<i>Callithrix</i> 4	10/29/29	Asibi	Injection of blood	<i>M. rhesus</i> R1	— 101.7	100.5 101.2	101.9 102.1	103.5 104.0	103.8 103.7	103.5 103.8	103.6 102.0	D				Died (yellow fever?)
<i>Callithrix</i> 7	11/12/29	Asibi	Mosquito bites Batch 248	<i>M. rhesus</i> R15	— 101.6	101.2 101.9	101.6 102.0	102.6 102.4	102.1 101.9	101.6 101.7	101.9 102.0	101.8 101.9	102.5 102.2	102.0 102.5	101.7 101.9	Recovered
<i>Callithrix</i> 8	11/12/29	S.R.	Mosquito bites Batch 245	<i>M. rhesus</i> R16	— 102.7	101.9 101.6	101.2 101.9	101.7 101.5	101.8 102.2	101.9 102.9	102.4 101.9	101.7 101.6	101.2 101.0	101.7 101.9	102.9 102.4	Recovered
<i>Callithrix</i> 9	11/22/29	S.R.	Mosquito bites Batches 245 and 249	<i>M. rhesus</i> R16 <i>Ateles</i> 3	— 101.7	101.9 101.5	101.7 102.0	101.9 101.7	102.0 102.8	D						Died as result of bleeding?
<i>Callithrix</i> 12	12/13/29	S.R.	Mosquito bites Batches 283 and 284	<i>M. rhesus</i> R17	101.6 102.2	102.5 102.4	102.7 103.5	101.9 103.1	103.0 102.5	102.4 102.9	102.7 102.5	102.9 103.0	102.7 102.9	102.5 103.0	103.7 102.9	Died as result of bleeding?
<i>Callithrix</i> 13	12/16/29	S.R.	Injection of blood	<i>Callithrix</i> 12	102.0 101.8	102.2 103.4	101.0 102.6	102.0 102.9	103.2 103.5	100.0 95.4						Died as result of bleeding?
<i>Callithrix</i> 15	12/21/29	S.R.	Injection of liver emulsion	<i>Callithrix</i> 13	— 102.0	102.4 102.1	102.5 102.7	102.6 102.4	102.5 102.5	102.4 103.0	102.9 103.2	103.7 103.6	103.4 103.5	103.6 103.4	D	Cause of death undetermined

Callithrix 17	12/26/29	Asibi	Mosquito bites Batches 275 and 276	Callithrix 4 <i>Leontocbus</i> 1	Died on 15th day, cause unknown									
					101.6	102.0	102.2	103.0	103.5	102.7	103.4	103.0	103.5	97.9
					101.9	101.8	101.9	102.5	102.9	103.7	102.9	102.6	103.5	102.0
Callithrix 18	12/30/29	Asibi	Injection of blood	Callithrix 17	—	103.1	102.2	103.0	103.4	103.5	103.6	103.8	103.9	104.0
					101.8	103.5	103.2	103.3	103.6	103.7	103.6	103.7	103.8	104.2
Callithrix 19	1/ 3/30	Asibi	Injection of blood	Callithrix 18	101.6	102.0	101.2	101.9	102.2	102.4	102.0	101.7	102.5	94.0
					101.9	101.9	101.8	102.0	102.1	102.2	101.9	102.1	99.7	D
Callithrix 20	1/ 8/30	Asibi	Injection of blood	Callithrix 19	101.9	102.2	102.0	104.0						
					102.1	102.0	105.5	94.0						
<i>Leontocbus</i> 1	11/ 2/29	Asibi	Injection of liver emul- sion	<i>M. rhesus</i> R12	—	102.2	103.6	104.0	103.7	103.6	104.2	103.8	102.9	D
					101.5	103.0	104.5	103.9	103.8	104.6	104.0	103.6	102.0	
<i>Leontocbus</i> 2	1/29/30	Asibi	Mosquito bites Batch 324	<i>M. rhesus</i> R11	—	102.1	103.5	104.4	103.6	103.6	103.4	103.9	104.8	103.9
					101.9	102.7	103.9	103.9	103.8	103.7	103.6	104.0	104.0	103.7
<i>Leontocbus</i> 3	2/ 1/30	Asibi	Injection of blood	<i>Leontocbus</i> 2	—	103.1	103.5	103.6	103.7	D				
					102.0	102.9	103.7	103.8	101.8					
<i>Leontocbus</i> 4	2/ 4/30	Asibi	Injection of blood	<i>Leontocbus</i> 3	—	102.8	104.8	104.4	104.5	104.0	104.2	103.9	103.1	102.2
					101.9	104.6	104.7	104.1	104.2	104.5	104.0	103.6	102.7	102.1
<i>Leontocbus</i> 5	2/ 5/30	Asibi	Injection of blood	<i>Leontocbus</i> 4	—	102.2	103.9	104.0	104.1	104.0	104.0	103.9	104.2	103.9
					100.9	103.0	104.2	103.9	104.0	104.2	104.1	104.0	104.1	102.2

or by mosquito feeding. However, after the virus was carried through a series of marmosets, it appeared to decrease in virulence for *M. rhesus*. This was not the case when the virus was passed through certain other species of Brazilian monkeys. A very few of the marmosets showed a definite fever (104°F. , or above). Most of them died while under observation, but the lesions found at necropsy did not resemble those of yellow fever as seen in humans, in the *rhesus* monkey, and in certain other species of monkeys.

Experiment I.—On October 29, 1929, Marmoset 4 was inoculated intraperitoneally with 2.5 cc. of citrated blood, containing Asibi strain virus, from *M. rhesus*



R1. The animal's initial temperature was 101.7° . On November 1 the temperature reached 104° , blood was transferred to *M. rhesus* R2, and mosquito Batch 275 was allowed to engorge. Marmoset 4 was found dead on November 4. There was a little dried blood about the nose and mouth. Jaundice was doubtful. The lungs showed a patchy congestion with, perhaps, a trace of bronchopneumonia. The liver had a slate-colored surface, but on section appeared yellow and unmistakably fatty; the organ was friable. The spleen was enlarged; its pulp was soft and its follicles were small. Kidney sections were opaque, with slight bile-staining and injection of the vessels. The gastro-intestinal tract was negative. Microscopically the liver showed degeneration, mainly midzonal, including a few necrotic cells. Fat was present in large amount, with the heaviest deposition in the midzones. A few leucocytes were noted. *M. rhesus* R2 died on the fifth day with yellow fever. Mosquito Batch 275 fed on *M. rhesus* R3 on November 21. This animal also died on the fifth day with typical yellow fever.

Experiment II.—On November 12, 1929, Marmoset 7 was fed upon by mosquito Batch 248, infected with Asibi strain virus. The initial temperature was 101.6° .

There was no subsequent fever and no significant rise in temperature (see table). However, on November 16, 1.5 cc. of blood was taken from the heart and injected into *M. rhesus* R4, which died on the sixth day with typical yellow fever.

Experiment III.—Marmoset 8 was fed upon November 12 by mosquito Batch 245, infected with the S.R. strain of virus. On the fourth day (November 16) blood was withdrawn for injection into *M. rhesus* R5. This monkey died on the fifth day with typical yellow fever. Marmoset 8 showed no significant rise in temperature at any time.

Experiment IV.—(Scheme I.) On November 22, 1929, Marmoset 9 was fed upon by mosquito Batches 245 and 249, infected with the S.R. strain of virus. The initial temperature was 101.7°. On November 26 the temperature in the afternoon was 102.8°. The animal was bled, and mosquito Batch 293 was allowed to engorge. On the following morning more blood was taken, and the monkey died soon thereafter. At necropsy the centers of the liver lobules showed as red dots surrounded by zones of an orange-yellow color; the organ was friable. The gastric mucosa showed a few dark streaks which appeared to be changed blood. The other organs revealed nothing of note. Microscopically, the lobules of the liver were seen to have a certain amount of midzonal degeneration, with deposition of fat, and perhaps a few necrotic cells. There was congestion, and many leucocytes were observed (mainly polymorphonuclear, occurring in small nests). The kidneys showed congestion and a slight cloudy swelling. On December 16 mosquito Batch 293 fed on *M. rhesus* R6, which died on the fifth day with typical yellow fever.

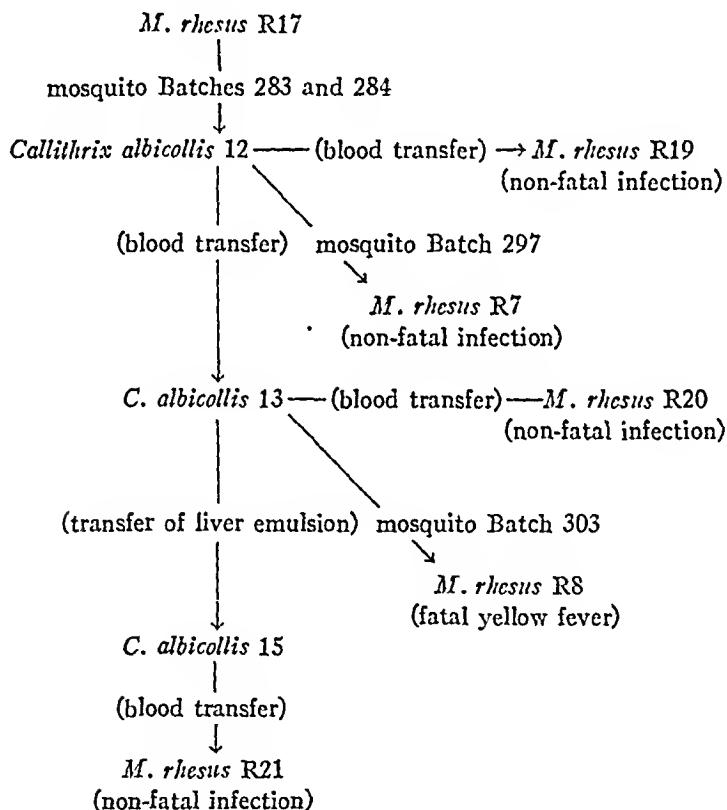
Experiment V.—(Scheme II.) This experiment consisted in the infection of Marmoset 12 by mosquitoes carrying the S.R. strain of virus, passage of the virus to Marmoset 13 by the injection of blood taken from Marmoset 12, and to Marmoset 15 by injection of emulsion of liver from Marmoset 13. Blood transfers to *rhesus* monkeys from Marmosets 12, 13, and 15 all produced definite infection, with fever and subsequent immunity to reinoculation, but no deaths. Mosquito transfer from Marmoset 12 to *M. rhesus* R7 was also non-fatal. However, mosquito Batch 303, infected on Marmoset 13, induced by its bites a fatal infection in *M. rhesus* R8. None of the marmosets had a definite fever (see table), and at necropsy none of them showed lesions suggestive of yellow fever.

Experiment VI.—(Scheme III.) In this experiment virus was passed to Marmoset 17 by mosquitoes, themselves infected on marmosets, and from Marmoset 17 through Marmosets 18 and 19 to Marmoset 20 by direct transfer of blood, taken in each case on the fourth day following inoculation. Blood from Marmoset 17 and the bites of mosquitoes fed on this animal caused fatal infections in *rhesus* monkeys. Blood virus from Marmoset 18 produced a fatal infection in *M. rhesus* R9. Blood from Marmosets 19 and 20 caused non-fatal infections in *M. rhesus* 10 and 11, and the bites of mosquito Batch 326, fed on Marmoset 20, produced non-fatal infections in two *M. rhesus*. Apparently the virus became attenuated for the *rhesus* monkeys by repeated passage through the marmosets. The last animal

in the series, Marmoset 20, had a clinical course suggestive of yellow fever. The initial temperature on January 8, 1930, was about 102°. On January 10 the temperature suddenly rose to 105.5°, at which time mosquitoes were fed and transfer of blood was made. On the afternoon of January 11 the temperature dropped to 94°, and the animal was killed. At necropsy there was a suggestion of icterus in the tarsal plates. The liver was a pale yellow; a small roundworm was found coiled up in one lobe. The other organs were essentially negative.

Scheme II

(S.R. strain of yellow fever virus)



Microscopical examination showed the liver to be nearly one half necrotic. The necrosis was primarily central, but extended into the midzones. There was a heavy leucocytic invasion. The kidneys showed a very slight cloudy swelling.

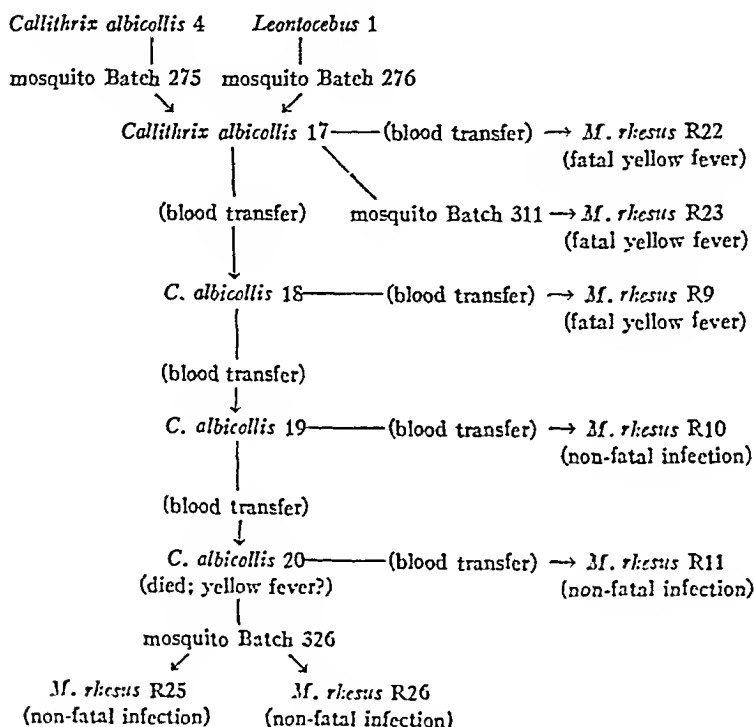
Genus *Leontocebus*. Tamarins. *Leontocebus ursulus* (Humboldt)

The little black tamarin, or marmoset *Leontocebus ursulus*, comes from Para. Elliot (5) calls it *Cercopithecus ursulus*. We were able

to secure five specimens for experimental work. These animals proved to be more susceptible to yellow fever virus than *C. albicollis*. Four of the five developed fever; all five died, two of them showing marked liver necrosis (Fig. 1), and two others the most intense jaundice that we have ever seen in monkeys.

Scheme III

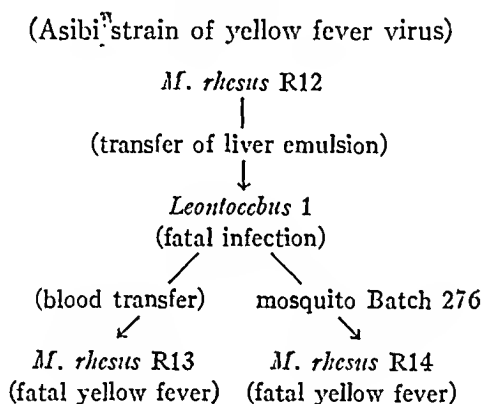
(Asibi strain of yellow fever virus)



Experiment VIII.—(Scheme IV.) On November 2, 1929, *L. ursinus* 1 was inoculated subcutaneously with 1.5 cc. of liver emulsion, containing Asibi strain virus, from *M. rhesus* R12. The initial temperature was 101.5°. On November 4 the temperature rose to 104.5°, at which time blood was transferred to *M. rhesus* R13 and mosquito Batch 276 was allowed to engorge. *M. rhesus* R13 died on the sixth day with typical yellow fever. Mosquito Batch 276 later induced a fatal attack of yellow fever in *M. rhesus* R14. *L. ursinus* 1 was found dead on No-

vember 11. Icterus was marked in the tarsal plates and in the intima of the aorta. A small abscess was found in the anterior abdominal wall at the point of injection of liver emulsion. The liver was rather friable, with sharp edges; the peritoneal surface was reddish brown, with purple discolorations in places. The cut surface was also reddish brown, with indistinct lobular markings. The spleen was swollen, with rounded edges, and was congested and purplish in color. The other organs did not appear abnormal in the gross. Microscopically, the liver showed a severe, but irregular, necrosis. In the midzones frequent islands and strands of cells were left intact. Necrosis sometimes extended to the central veins. Near the capsule several areas were noted where necrosis was complete and digestion advanced.

Scheme IV

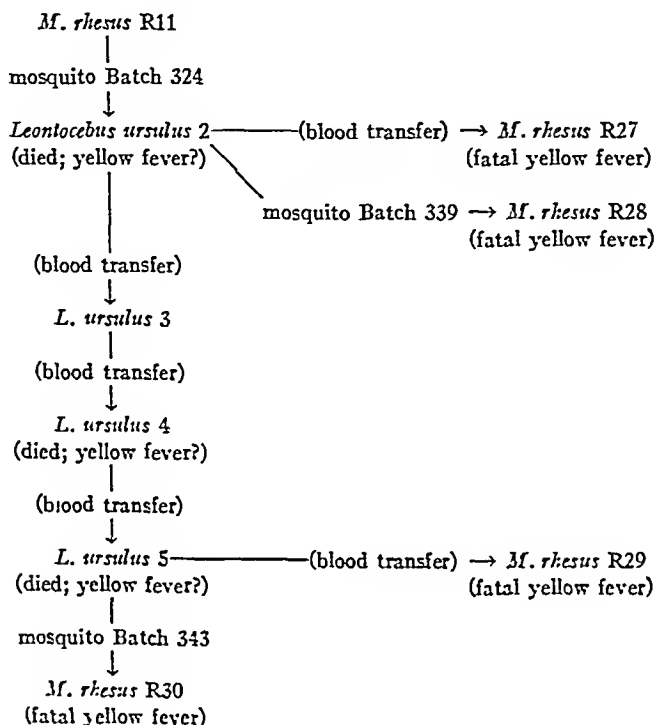


Experiment VIII.—(Scheme V.) On January 29, 1930, mosquito Batch 324, infected with Asibi strain virus, fed upon *L. ursulus* 2. The strain was passed in succession through *L. ursulus* 2, 3, 4, and 5 and back to *M. rhesus* from the last marmoset by blood transfer and by mosquito bites, producing fatal infections. Transfers from *L. ursulus* 2 also induced fatal infections in *rhesus* monkeys; no transmission to *M. rhesus* was attempted from *L. ursulus* 3 and 4. The temperature reactions are given in the table. Although intense icterus was noted at autopsy in *L. ursulus* 4 and 5, the most remarkable lesions were found in *L. ursulus* 2. This animal had fever on February 1 and again on February 5 and 6. It died on February 11. Marked icterus was noted in the tarsal plates, in the intima of the aorta, and even in the skin. The liver was friable and of a curiously mottled yellow, orange, and brown (in general, lighter than normal). The spleen appeared to be swollen. The kidneys were pale and bile-stained. The stomach contained food mixed with a considerable amount of changed blood. Microscopically, the liver showed over 80 per cent necrosis, essentially midzonal, with rings of cells, relatively unaffected (except for fat), around the vessels. Occasional nests of intact cells were also found in the midzones. The necrotic cells showed very

eosinophilic cytoplasm and nuclei in various stages of degeneration and fragmentation. There was congestion of vessels, generalized pigmentation, and deposition of fat (especially in cells around the central veins). Round-cell infiltration and hyperplasia of epithelium of the bile-ducts was noted in the portal spaces. The kidneys showed slight cloudy swelling and injection of the glomeruli.

Scheme V

(Asibi strain of yellow fever virus)



DISCUSSION

The experiments here presented show that yellow fever virus can be introduced into the marmosets, *Callithrix albicollis* and *Leontocebus ursulus*, by mosquito bites, that it can be passed through at least four animals and can be taken out again by mosquitoes which subsequently are able to transmit the infection to *rhesus* monkeys. There is some evidence that in such a series of passages through *C. albicollis*

the virus becomes somewhat attenuated for *M. rhesus*. Apparently no change in the virus takes place in passage through *L. ursulus*.

From five *C. albicollis*, numbers 3, 5, 7, 8, and 18, convalescent serum was used in protection tests against Asibi strain virus. Protection was secured in every instance with these convalescent sera, but not with serum from a normal marmoset. Numbers 7, 8, and 18 are mentioned in the protocols. Numbers 3 and 5 were inoculated with blood containing virus but neither showed a febrile reaction; no attempt was made to transfer the virus from them to other monkeys.

The lesions found at necropsy in *C. albicollis* did not closely resemble those found in human beings or *rhesus* monkeys that had died of yellow fever. On the other hand, the liver lesions in two of the five *L. ursulus* were very extensive and comparable to the usual midzonal necrosis of susceptible species.

The fact that the virus of yellow fever can be passed through marmosets with comparative ease may have some importance in the epidemiology of the disease in nature. These little monkeys are frequently kept as household pets, and even the wild ones are often found on uncleared land within urban limits. It is conceivable that they might pick up yellow fever from domestic sources, carry it to outlying districts, and aid in its wide dissemination. Such a course of events seems the more possible since we know that various "wild" species of mosquitoes are fairly efficient vectors of the virus.

SUMMARY AND CONCLUSIONS

1. It has been possible to introduce yellow fever virus into the small Brazilian monkeys, *Callithrix albicollis* and *Leontocebus ursulus*, by the bites of infected mosquitoes and to carry the virus through a series of four passages in each species and back to *rhesus* monkeys by the bites of *Stegomyia* mosquitoes fed on the last marmoset of each series.

2. Five specimens of *L. ursulus* were used. Four developed fever, and all died during the experiments. At least two showed liver necroses comparable to those found in human beings and *rhesus* monkeys that died of yellow fever.

3. Twenty specimens of *C. albicollis* were used. Very few showed a

temperature reaction following the introduction of virus. Of those that died, none had lesions typical of yellow fever as seen in certain other species of monkeys and in humans.

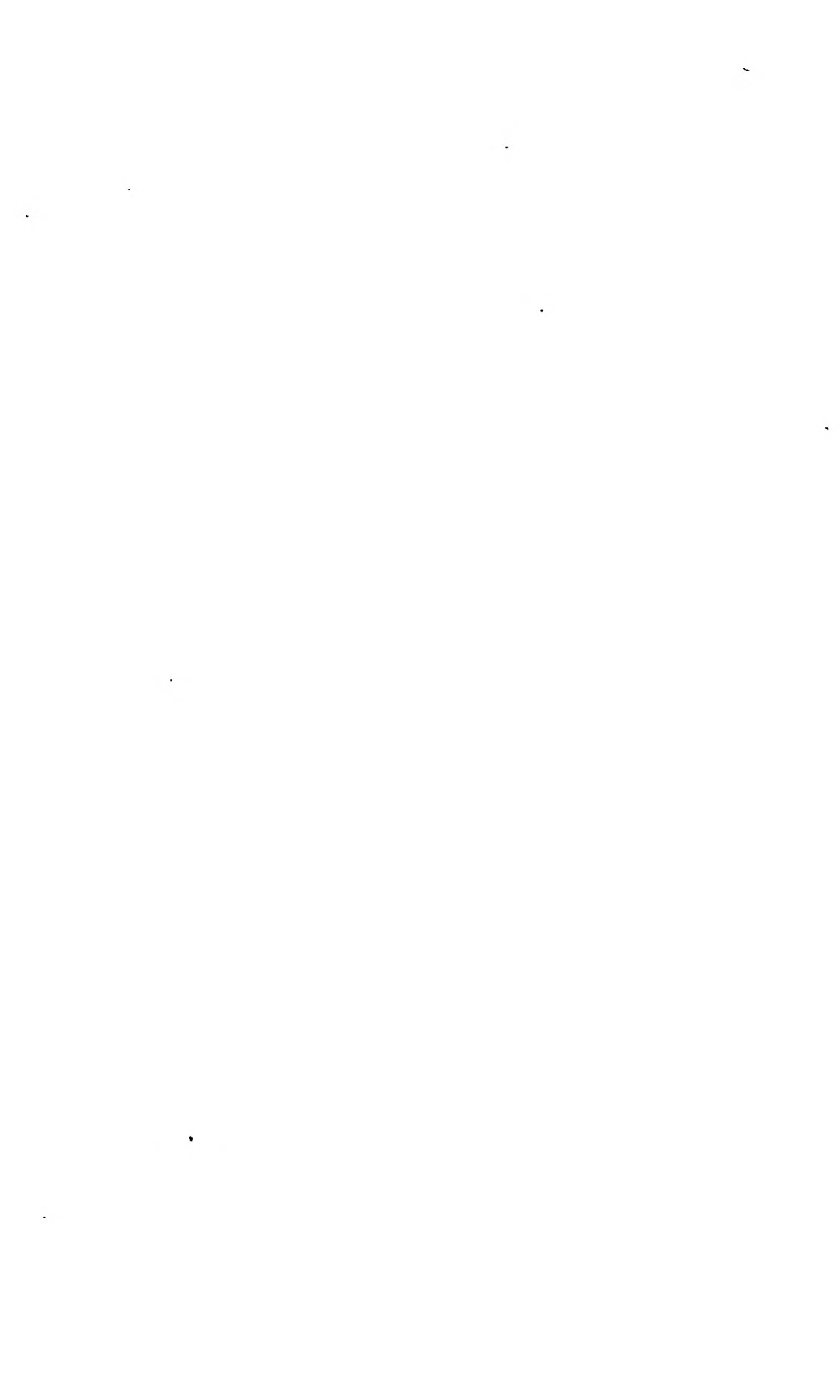
4. The convalescent serum from each of five *C. albicollis* protected a *rhesus* monkey against yellow fever virus, but the serum from a normal marmoset of the same species was found to be non-protective.

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EXPLANATION OF PLATE 16

FIG. 1. Section of the liver of a black marmoset which died of yellow fever. The tissue shows marked necrosis. ($\times 320$.)



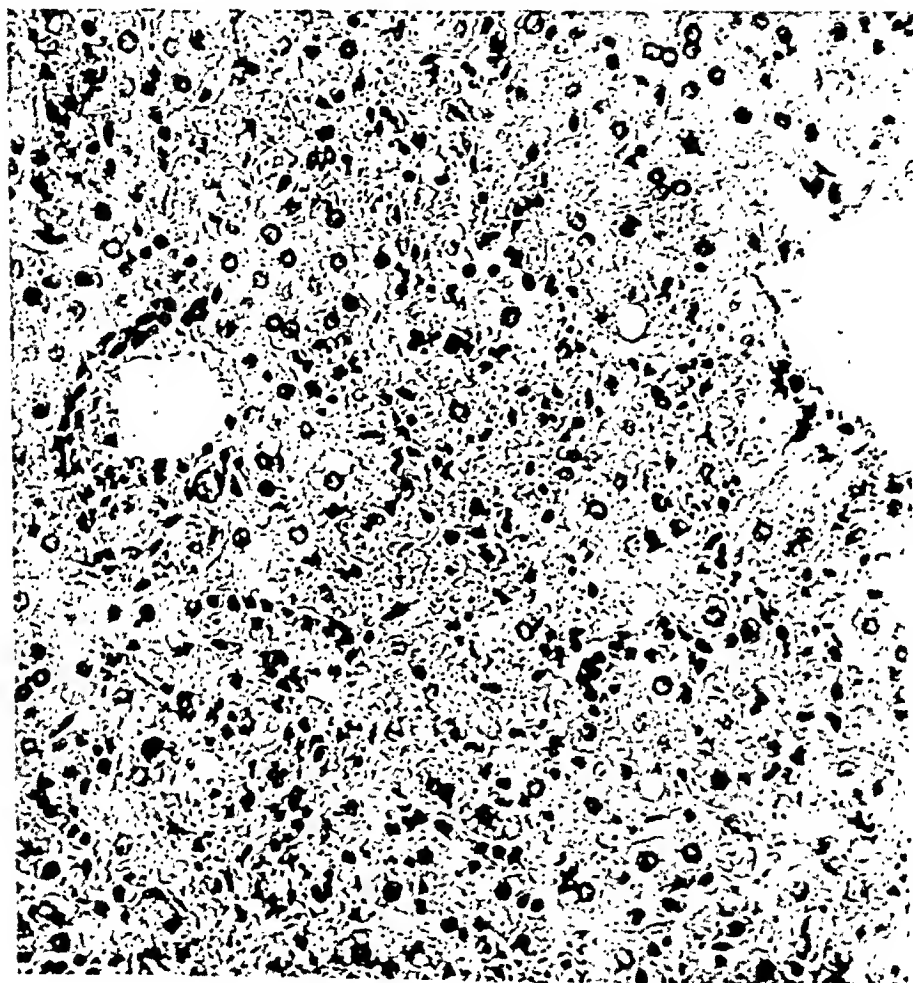


FIG. 1

(Duke University) by the gift of Dr. J. H. R. Taylor

THE EXTRACTION OF THE CELL CONTENT OF MICRO-ORGANISMS BY PERCUSSION OF THE FROZEN CELLS

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INTRODUCTION

For many purposes it is desirable to obtain extracts of microorganisms without delay and free from the changes which undoubtedly accompany the procedures now used. The present methods involve chemical agents, autolysis, prolonged alternate freezing and thawing, or dessication and grinding. These processes often bring about changes which may effect the nature of the extract. The method of the Buchner press, which has been used for obtaining yeast extracts, while avoiding some of the objectionable features of other procedures is apparently not applicable for general use with pathogenic bacteria. It occurred to us that if microorganisms were frozen very quickly so that the ice crystals were very fine, and if this mass of cells containing the fine crystals were then subjected to sudden high pressure, such as may be obtained by blows applied to a piston in a closed cylinder, the friction of the ice crystals might either disrupt the cells or so change the character of the membrane that the intra-cellular material would be forced out. The apparatus described here was designed to test this idea, and as will be seen, its use has given encouraging results.

Apparatus and Materials

The apparatus employed consisted of a cylindrical steel chamber and a closely fitting steel plunger so cut as to move freely without compression. The chamber was made by cutting a hole, 1.6 cm. in diameter and 8.5 cm. deep in a piece of steel shafting 10 cm. in diameter and 12.5 cm. long, with a machine cutting tool. The chamber was flat at the bottom. About 1 cm. from the top of this chamber a cir-

cular groove was cut to form an overflow chamber having a capacity of about 6 cm. The plunger was made of high grade steel tempered at the lower end.

The cells which were used for extraction were pure cultures of bakers' yeast, 24-hour broth cultures of *B. coli*, *B. xerosis*, *Streptococcus hemolyticus*, Pneumococcus Type II, a 5-hour dextrose broth culture of Pneumococcus Type I, 4-day cultures of *B. dysphtheriae* and a 5-day glycerine bouillon culture of the bovine tubercle bacillus. The cells were repeatedly washed with distilled water until the supernatant obtained by centrifuging no longer gave the biuret reaction for proteins and were then suspended in distilled water.

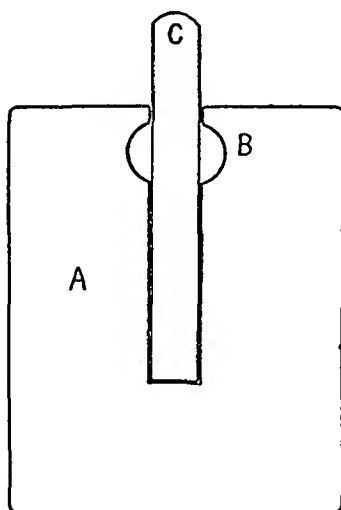


FIG. 1. Extraction apparatus consisting of a steel chamber, A, having a reservoir, B, and fitted with a tempered steel plunger, C.

EXPERIMENTAL

Before each experiment the apparatus was cleaned by scouring and washing and sterilized in a hot air oven. It was then cooled, first by packing with a salt and ice mixture and then by placing into an insulated container and packing with solid CO₂ (dry ice). The chamber was then charged with 2 cc. of the suspension of cells and the plunger replaced and fastened with a rubber collar so as to remain suspended about 1 cm. above the charge. The top of the apparatus was then covered with sterile cotton. After 20 minutes additional cooling in dry ice, the apparatus, along with its insulated container, was placed on a heavy metal base and the plunger struck twenty-five heavy blows with an 8-pound hammer. This subjected the cells to the crushing action of the ice crystals. Intervals of 10-seconds or longer between blows were allowed to prevent the charge from being forced into the overflow chamber by the melting effect of pressure and heat before the desired number

of blows had been struck. The charge was thawed, by placing the apparatus in a water bath, and was then removed with a pipette. After centrifuging, the supernatant liquor was passed through a Berkefeld filter.

All of the supernatants and filtrates obtained by subjecting the various microorganisms to this treatment were slightly opalescent, frothed readily and gave a heavy precipitate when a few drops of 10 per cent acetic acid were added. They always gave the biuret reaction for proteins but generally only a weak Molisch test for carbohydrates. All of them became more opalescent when boiled and in some cases a slight precipitate was obtained. For control in each case a portion of the same suspensions of the microorganisms used in the experiments was frozen with dry ice, thawed, and the supernatants, obtained by centrifuging, tested with the biuret and acetic acid precipitation tests. In every instance these tests on the controls gave negative results.

Morphologic evidence of cell destruction was obtained in case of yeast cells and pneumococci. Practically all of the yeast cells were found to be without vacuoles after percussion. There was also some evidence of cell fragmentation. *Pneumococcus* cells which before treatment were Gram positive, after treatment showed many Gram negative cells with débris. After a second treatment of these same cells only occasional whole cells were visible. The preparation showed a large amount of Gram negative cell detritus.

Gram stain preparations of *B. xerosis*, *B. dyphtheriae* and hemolytic streptococcus gave unconvincing evidence of any change in staining reaction or morphology. The acid fast character of the bacillus of bovine tuberculosis was not found to be changed.

DISCUSSION

The microorganisms used in this investigation were chosen with respect to their varied resistance to other methods of extraction. The uniformly positive results which were obtained indicate that this method should have a wide range of application in obtaining similar extracts from other species of resistant organisms. The treatment of frozen cells by percussion should facilitate further extraction of cell residues by chemical means.

The chief advantages of this method, however, lie in the possibility

of obtaining for immunological and biochemical studies fresh extracts of living cells, which have been kept at low temperature, thus minimizing the changes brought about by autolysis, and which are free from chemical contamination.

SUMMARY

A method is described for rapidly obtaining fresh extracts of microorganisms by percussion of the frozen cells.

Filtrates giving the biuret reaction and yielding heavy precipitates upon the addition of acetic acid were obtained from the washed cells of cultures of yeast, hemolytic streptococcus, pneumococcus, *B. coli*, *B. xerosis*, *B. diphtheriae* and the bacillus of bovine tuberculosis.

THE RELATION OF NATURAL HUMORAL ANTIPNEUMOCOCCAL IMMUNITY TO THE INCEPTION OF LOBAR PNEUMONIA

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Further progress in our understanding of lobar pneumonia in man not improbably depends to a great extent upon the determination of those conditions necessary for the inception of the disease, concerning which information is at present scarcely more than speculative. While the implantation of pathogenic pneumococci* by inhalation is presumably essential for the production of the pneumonic lesion, it does not seem likely that this is usually the deciding factor, for the reason chiefly that contact infection occurs so infrequently. Protection from infection may be due in part to the barrier interposed by the devious channels of the upper respiratory tract to the passage of the pneumococcus-containing droplets, but when the concentration and fineness of the droplets is sufficient, some of them reach the lung, as can be shown experimentally. Disturbed function of the respiratory tract accompanying mild infection of the upper or lower respiratory passages or following operations, especially on the upper abdomen, precedes the onset in a considerable percentage of pneumonia cases. In others with an antecedent chilling a general alteration in susceptibility may possibly take place as well. However, for those numerous instances in which pneumonia occurs suddenly during a period of apparent normal health another explanation must be sought.

Blake and Cecil's work on the experimental production of the disease in monkeys (1) suggests that in order to initiate lobar pneumonia it is

* Since pneumococci even of low virulence are capable of causing pneumonia under certain conditions provided they are "S" producing, it seems proper to term all such pneumococci as pathogenic.

only necessary to implant in the lungs a sufficient number of pneumococci of adequate virulence for the animal. In their experiments the number of organisms employed was very small. But other authors (2), working with dogs which possess much higher natural antipneumococcus immunity than the species of monkeys used above, have found that the simple intrapulmonary implantation of pneumococci either failed to cause pneumonia or produced the disease only if used in quantities enormously greater than could possibly occur in the natural acquisition of the disease in man. Is the difference in the ease with which lobar pneumonia can be induced in these two animal species due simply to their relative susceptibility to pneumococcus infection?

A previous study on the resistance of normal human beings to recently isolated strains of pathogenic pneumococci (3) has indicated that as a group they possess considerable natural immunity to these organisms as shown by the pneumococcus-destroying power of their blood which in degree is not much below that of the dog. But individuals were found to vary greatly in their reaction toward the several types of pneumococci. This ranged from marked killing power of the blood for one type to none against others. The fluctuations in antipneumococcus action were found to reside in the serum, not in differences in the leucocytes. These findings indicated the feasibility of securing further information about one of the factors which is possibly concerned in the inception of lobar pneumonia, namely the amount of circulating specific antipneumococcus substances at the onset of the disease.

Methods

The pneumococcal tests with human serum and leucocytes were carried out as previously described (4, 5). Serum samples were secured from the patient on admission and daily thereafter throughout the course of the disease. These were kept in an atmosphere of CO₂ at 4°C. and all were tested at one time.* Frequently in early cases the serum was tested as soon as the homologous pneumococcus was isolated. In every instance the experiments were performed with the organism isolated from the patient's lung, blood or sputum, and a control consisting of the pooled serum of four to seven normal individuals was run at the same time. The smallest amount of pneumococcus suspension used was 10⁻⁷ in all but cases V. C.

* Serum preserved in this way showed no deterioration in its pneumococcal-promoting properties for a period of 6 to 7 days.

and H. F. P. in which 10^{-8} was employed. Quantities less than 10^{-7} not infrequently led to irregularities in the test. In a number of cases the opsonic and mouse protective properties of the serum were determined also. In almost all instances the tests were repeated in order to verify the results. Blood cultures were taken in the usual manner, 6 to 10 cc. into broth and 1 cc. and 2 cc. into plates.

Clinical Cases

The present study comprises twelve cases of lobar pneumonia in whom observations were begun from 4 to 48 hours after the onset of the disease. The antipneumococcus properties exhibited by the serum within this period may be considered with a fair degree of certainty as referable to the class of natural immune substances, since acquired immune bodies have not been observed by us to develop before the third day of the disease.* The results of the tests as summarized in Table I revealed the unexpected finding that in the majority of cases the initial serum specimens possessed pneumococcal-promoting properties, sometimes slight and again as great as in normal individuals.** In only four cases was this property absent. The possibility that an organism other than that causing the disease was responsible for the serum differences, and in particular the exhibition of well marked antipneumococcus properties, is excluded by the fact that pneumococci isolated from the lung and blood stream in certain cases were affected to as pronounced a degree as were the organisms obtained from the sputum in others. Nor did the virulence of the pneumococcus strains appear to bear any relationship to the action on them of the patient's serum. Organisms against which one patient's serum-leucocyte mixtures showed marked killing action were fully as virulent when tested by animal inoculation and growth capacity in normal pooled human serum and leucocytes, as were those on which another patient's serum had no effect. That is, the variations appeared to reside in the individual rather than in the several pneumococcus strains. Again, the initial pneumococcal-promoting properties of the serum could not be associated with the extent of the lung lesion, as determined by

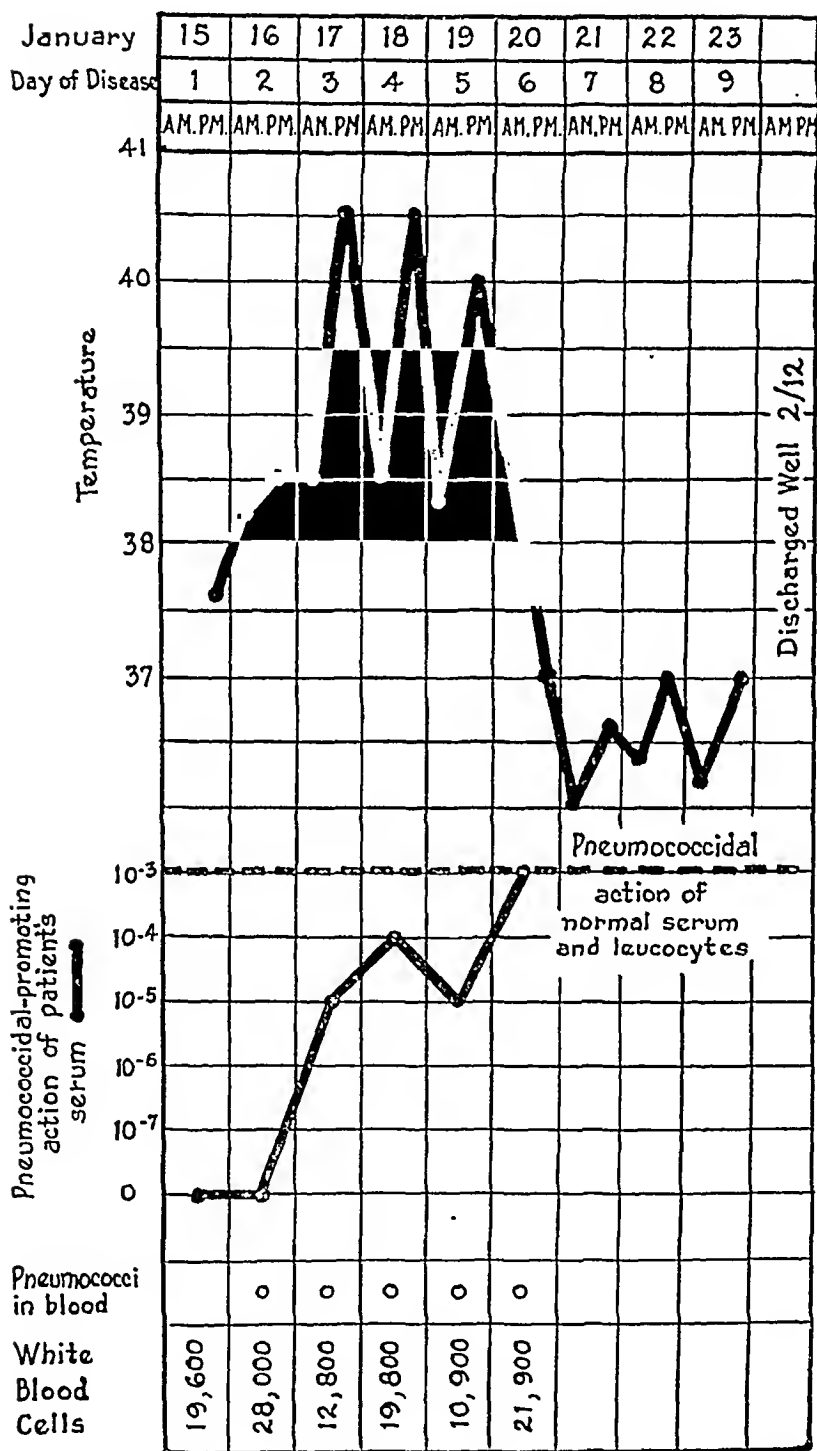
* As will be shown in a succeeding communication, acquired humoral immunity does not usually occur before the fourth or fifth day of the disease.

** By normal degree is meant the pneumococcal-promoting action of pooled normal human serum for the particular pneumococcus in question.

E. J.	IV	Sputum	No. Pn. killed Pn. in blood	10^{-6} —	10^{-6} —	10^{-3} —	10^{-5} —	10^{-4}				Rec'd 5th day
H. C.	III	Blood	No. Pn. killed Pn. in blood	10^{-7} +	10^{-7} +	10^{-6} 0	10^{-3} 0					Rec'd 4th day
J. V.	II	Blood	No. Pn. killed Pn. in blood	0 +	0 +							Died in 48 hrs.
F.	IIa	Sputum	No. Pn. killed Pn. in blood	10^{-4} 0	10^{-3} 0	10^{-3} 0	10^{-6} 0	10^{-4} 0	10^{-4} 0	10^{-4} 0		Rec'd 7th to 8th day
H. F. P.	IV	Lung	No. Pn. killed Pn. in blood	10^{-5} 0	10^{-6} 0	10^{-7} +	10^{-7} +	10^{-3} +	10^{-3} +	10^{-7} +	10^{-3} +	Died 8th day
V. C.	III	Blood	No. Pn. killed Pn. in blood	10^{-4} 0	0 +	0 +	0 +	+	+	+	+	Died 6th day

The minus powers of 10 above, represent the amounts of the standard pneumococcus containing 1 billion pneumococci per cubic centimeter which were killed by the serum and leucocytes obtained from approximately 0.5 cc. of blood.

— " Not done.



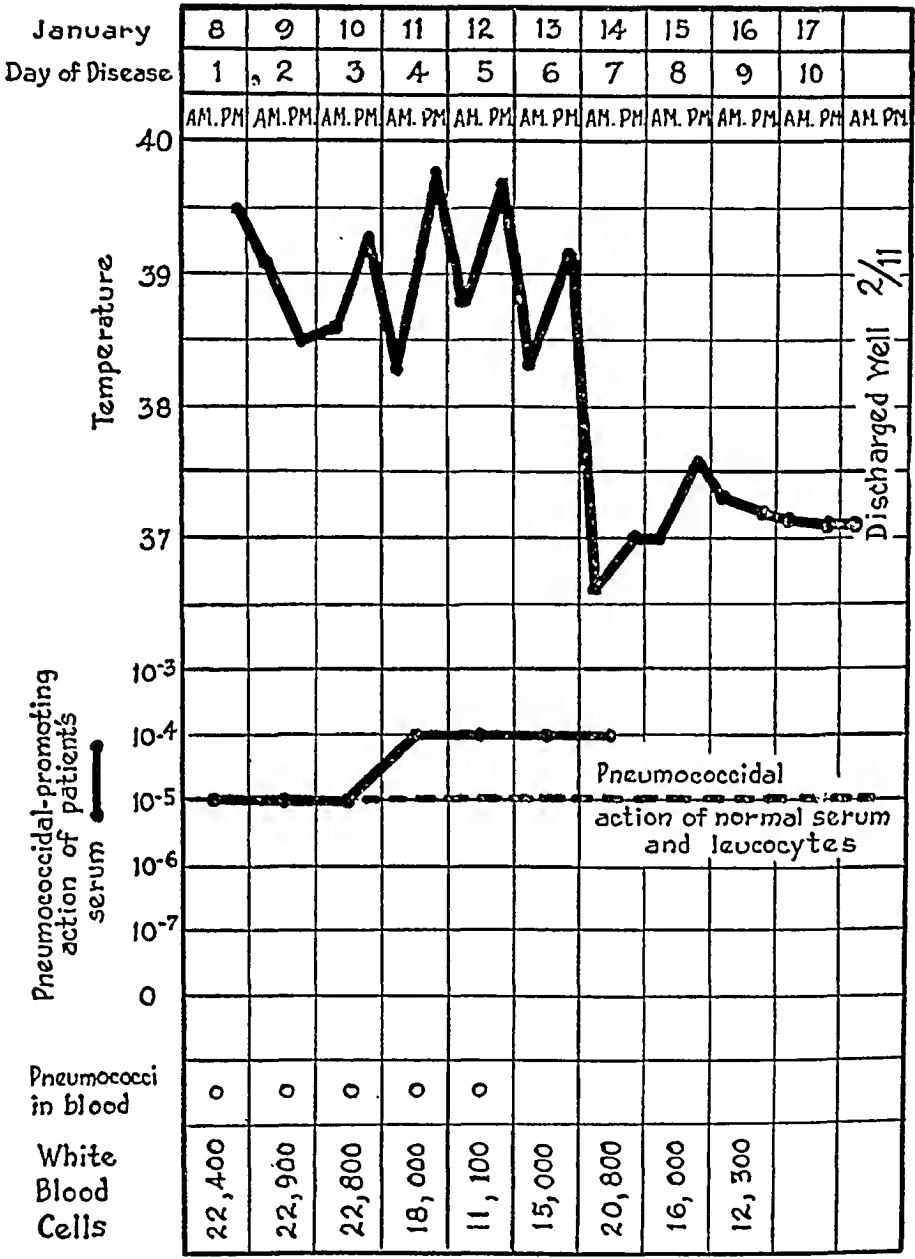
TEXT-FIG. 1. Case A. L., lobar pneumonia—Pneumococcus Type I.

X-ray and physical signs. Both cases A. L. and J. T. A., in whom a very small lesion existed at the time of the first observations, showed no pneumococcus immune properties in the blood serum. On the other hand, patients K. H., V. C. and H. F. P. showed lesions of considerable extent and a normal or at most a slight reduction in degree of pneumococcus-killing power in their blood. The number of cases is far too limited to permit of any conclusions in relation to pneumococcus type.

With the progress of the disease, changes in the pneumococcidal-promoting action of the serum usually followed one of several fairly regular courses. When antipneumococcus properties were not demonstrable at the beginning, none appeared until the onset of acquired immune-body production. Case A. L., Text-fig. 1, is an example. There were a number of other patients concerning whom data are not given in these tables (since observations were not begun until after 48 hours) whose blood serum exhibited the same findings.* In contrast were patients such as case K. H. (Text-fig. 2), who showed a well marked degree of pneumococcidal-promoting serum power all through the disease. A third course is shown by case V. C., Text-fig. 3. Following an initial normal titer of antipneumococcus properties there occurred a progressive and sometimes very rapid disappearance of the serum effect which did not reappear unless recovery took place. Of course all the patients studied did not fit exactly into these three categories, case H. C., for example, being an exception but they could practically all be classified as modification of one of them.

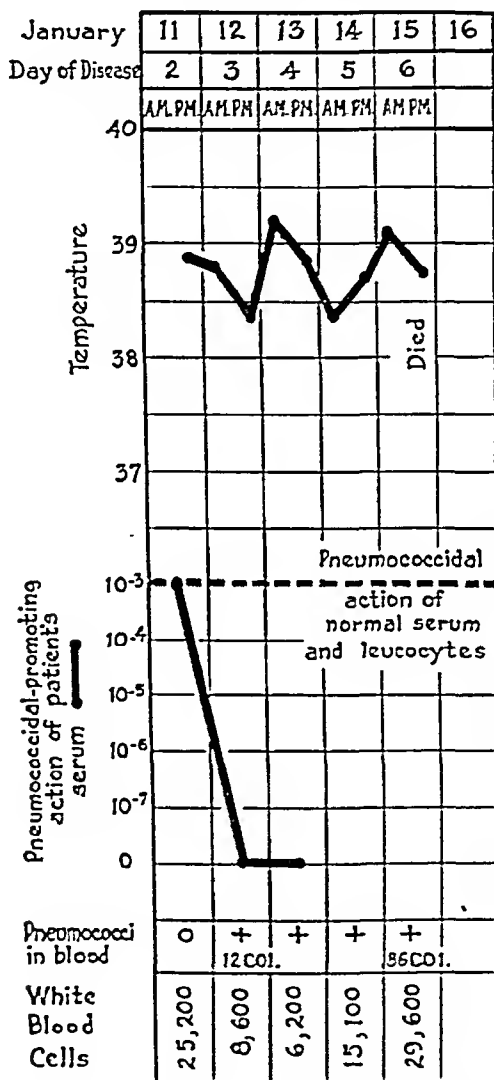
A constant relationship was found to exist between the concentration of immune properties in the serum and blood invasion. In the presence of a killing power of the serum and leucocytes represented by the destruction of as small an amount as 10^{-6} of the standard pneumococcus suspension, pneumococci were not found in the blood stream. When pneumococcidal action fell below this point blood invasion tended to occur. That it did not always occur even when antipneumococcus properties were not demonstrable in the serum may be taken as an indication that a modicum of this action still persisted. Indeed when the tests were carried out with numbers of pneumococci as few as 10^{-8} of the suspension (containing actually less than 10 pneumococci)

* The details of the studies on these patients will be given in a following communication.



TEXT-FIG. 2. Case K. H., lobar pneumonia—Pneumococcus Type III.

a residuum of pneumococcus-killing power was sometimes found to persist even in the presence of an increasing blood invasion, as in the instance of case H. F. P. Probably other factors participate in con-



TEXT-FIG. 3. Case V. C., lobar pneumonia—Pneumococcus Type III.

trolling the penetration of pneumococci into the blood channels, such as the degree and perhaps nature of the cellular reaction in and surrounding the pneumonic lesion, but the pneumococcal action of the blood would seem to be the one of chief importance.

Because of the observations of other workers who have studied the immune properties of the serum during lobar pneumonia by means of mouse protection, a word should be said concerning the relative sensitiveness of the pneumococcal reaction as carried out in the present investigation and the mouse test. A comparison of these two techniques on the same serum specimens has shown the pneumococcal test to be the more sensitive. A serum which causes killing of pneumococcus in the serum-leucocyte mixture in quantities as large as 10^{-5} and 10^{-6} of the standard suspension will often fail to cause protection of mice against amounts greater than 10^{-7} of the same suspension or exert no protection at all. Hence it is not surprising that most workers have failed to find mouse protective properties in the serum during any but the later stages of the disease or have found them only to a degree that seemed negligible on account of the individual variations exhibited by mice.

DISCUSSION

The findings at once suggest the question, does the presence of pneumococcal properties in the blood after the onset of the disease indicate that they were there just prior to or at the inception of the pneumonic process. While no direct answer can be given, observations on the experimental disease in animals and on human beings with pneumonia afford data for certain assumptions in the affirmative. Marked and rapid fluctuation in the degree of natural pneumococcal activity of the blood has not been noted in either man or animals during health or in disease. In the experimental animal, as one of us has shown (6), a generalized pneumococcus infection is accompanied by a pronounced and progressive diminution in circulating antipneumococcus immunity which does not reappear until the development of acquired immune substances occurs coincidentally with recovery. However, when the pneumonia process is localized as in experimental lobar pneumonia, the natural humoral immune bodies tend to persist with little diminution throughout the course of the disease. Blood

samples taken immediately before infection is initiated show a normal pneumococcal action. Tests on pneumonic patients indicate that once a marked decrease or disappearance of the antipneumococcus properties of the blood has occurred they do not show an increase again until shortly before or at the time of recovery.

Other workers have also observed evidence of natural antipneumococcal immunity early in the course of lobar pneumonia. Park and Cooper (7) found in more than a third of pneumonia patients they studied appreciable mouse protective action of the serum on the second day of the disease. In a paper just published Ward (8) states briefly that he found the defibrinated blood of pneumonic patients early in the disease to possess a normal pneumococcal action against the causative organism. Details of the cases and tests are not given.

If pneumonia can develop in the presence of a normal circulating antipneumococcus defense mechanism, as seems evident from the above observations, what is the actual mechanism of infection? The most reasonable explanation would seem to be that local changes take place of such nature as to provide conditions for the growth of the pneumococci while protecting them from the pneumococcal action of the blood. There might conceivably be failure of the normal eliminatory mechanism, tissue injury by concurrent infection, allergic reactions, or a combination of these factors. Once pneumococcus growth is established, and perhaps this may be entirely within the lumen of the bronchus—the constant diffusion of bacterial metabolic products in a circumscribed area would produce increasing tissue injury, one of the results of which has been shown to be deposition of fibrin in the capillaries and lymphatics. This would tend further to protect the pneumococci from the full action of the circulatory immune bodies. How early and to what extent the impairment of the circulation occurs in lobar pneumonia in man is not definitely known. The experimental observation of Kline and Winternitz (9), however, would suggest that it takes place early in the disease course and becomes pronounced as the lesion progresses.

The hypothesis has recently been put forward by Coryllos and Birnbaum (10) that lobar pneumonia is initiated as a result of extensive atelectasis produced by occlusion of a large bronchus. In order that the condition may occur the mucous plug must exclude

completely the passage of air and persist for some time. Pneumococci distal to the plug are thus protected from elimination. It is not our desire to enter upon a detailed discussion of this view,* but it should be pointed out that certain of the assumptions upon which the hypothesis rests are not generally supported by clinical evidence. According to Coryllos and Birnbaum, the early signs of the pneumonic lesion are found at the periphery where atelectasis occurs first, the exudative process extending progressively from the hilum to the periphery. As a matter of fact, X-ray studies in the early part of the disease show, in a large percentage of cases of lobar pneumonia, a shadow confined to the hilum which gradually spreads out to the periphery as the disease evolves. Furthermore, in none of our early cases have we found evidence of atelectasis on the affected side either by physical signs or X-ray. However, aside from the question of atelectasis and complete plugging of a bronchus, a local failure of the normal eliminatory mechanism (implied in the hypothesis of Coryllos and Birnbaum) which leads to stasis of bronchial secretion might provide, as suggested above, the environmental state necessary for effective action of the implanted pneumococci.

The rôle played by the natural circulatory immune bodies in the process of localization would seem to be to destroy pneumococci at the periphery of the lesion, thus retarding the spread of the process and limiting or preventing blood invasion. With an initial marked reduction or absence of these humoral immune factors in the presence of an infecting organism of high virulence a rapid spread of the pneumonic process and a generalized infection might be expected to occur. Whether patients such as case J. V., who died within 48 hours of an overwhelming infection were deficient in natural antipneumococcus substances at the inception of infection, can only be surmised until further data are available, but the sequence of events would suggest that such was the case.

SUMMARY AND CONCLUSIONS

A study of the pneumococcal-promoting action of the serum of lobar pneumonia patients, secured from 4 to 48 hours after the onset of

* The subject will be considered fully in a subsequent communication on the production of experimental lobar pneumonia.

the disease, has revealed the fact that in the majority of instances the serum possessed the power to promote killing of the homologous pneumococcus, isolated in different instances from the lung, blood, and sputum. While in some instances this action was slight, in others it was present to as great a degree as in normal individuals and persisted as long as 48 hours or more after the beginning of the disease. The variations observed from case to case were not related to the extent of the pneumonic lesion or to the virulence of the several pneumococcus strains but appeared to depend on differences in individual human beings in respect to the natural antipneumococcus properties of their blood and their reaction to the invading microorganism. A constant relationship was found to exist between the concentration of immune properties in the serum and blood invasion. In the presence of a well marked pneumococcal-promoting power pneumococci were not found in the blood stream, and only when this property was greatly diminished or lost did blood invasion occur.

The findings which are supported by certain previous experimental observations, indicate that lobar pneumonia can occur in the presence of a normal circulating antipneumococcus defense mechanism. From this it is inferred that before pneumococcus growth can be initiated there must be present in the lung local changes of such nature as to provide conditions for the multiplication of pneumococci protected from the pneumococcal action of the blood. Suppositions as to the nature of these changes and the establishment of the pneumonic lesion are discussed.

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THE KILLING OF CERTAIN BACTERIA BY X-RAYS

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PLATE 17

(Received for publication, May 24, 1930)

The experiments to be described are an extension to soft X-rays of those¹ previously made with cathode rays. They consist essentially of studies of the time rate of killing of single bacteria by the general radiation from a tungsten tube operated at low voltage and by the characteristic K-radiation of copper.

EXPERIMENTAL

The organisms *B. coli* and *B. aertryke* were chosen for these observations because they readily give the distribution of single cells that is needed if statistical counts are to have meaning. The bacteriological procedures were identical with those already described. As before, standard cultures were provided by Dr. L. T. Webster of this Institute. Spreads of single bacteria upon the surface of agar (ca 200 organisms per in.²) were irradiated before multiplication could take place. After incubation, counts were made of the number of colonies growing out upon a stamped irradiated area and upon an equal and adjacent control area marked at the time of irradiation. From these data survival ratios have been calculated which, together with measurements of the X-ray intensity striking the agar plate, can be made to give information concerning details of the killing action of the rays. In order that the survival ratios obtained in this way should refer to enough organisms to be statistically significant, results from many plates have been averaged.

In the first group of experiments the X-rays used were the unfiltered general radiation from a Siemens Bucky tube having a tungsten target and a Lindemann glass window. The voltage across this tube was ca 12 KV peak and was provided by the unrectified output of a suitable transformer. A steady current of 8 MA was obtained by heating the

¹ Wyckoff, R. W. G., and Rivers, T. M., *J. Exp. Med.*, 1930, 51, 921.

cathode filament with an insulated storage battery. The bacteria were distant approximately 8 cm. from the tungsten target. Because of the long exposures required to give a sufficient killing action, only about 25 plates were irradiated in a day. Since the ratios of Table I and Text-fig. 1 are each averages of the counts upon 20 to 60 plates, several days were devoted to each experiment. These results obviously fall upon straight lines when plotted on semilogarithmic paper.

TABLE I
Survival Ratios with Bucky Tube

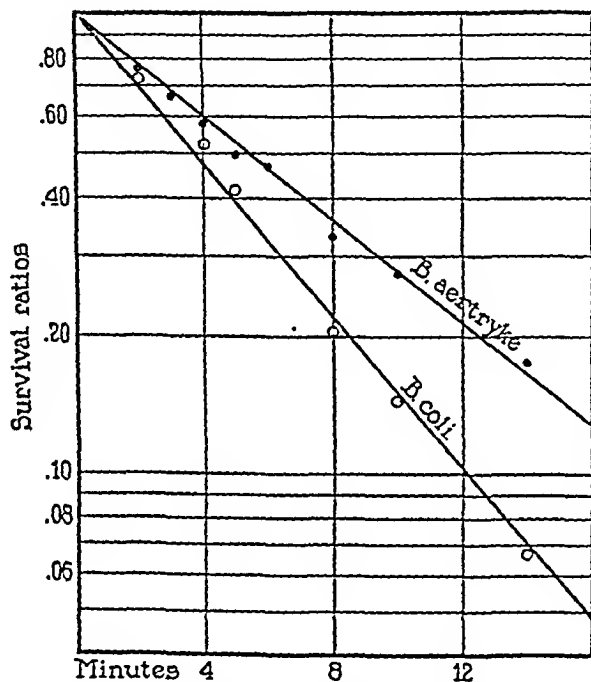
Time	Survival ratios	
	<i>B. coli</i>	<i>B. aertryke</i>
2 min.	0.750	0.768
3 "	—	.661
4 "	.526	.578
5 "	.416	.490
6 "	—	.466
8 "	.201	.329
10 "	.146	.274
12 "	.087	—
14 "	.064	.175

The survival ratios must therefore be expressed by an equation of the type

$$\frac{A_1}{A_0} = e^{-at}$$

Estimates of the intensity of the X-ray beam were made by measuring with a suitable chamber the ionization of air at the position of the irradiated bacteria. Subsequent experience has indicated that saturation currents were not obtained in these first experiments. No new determinations have been carried out because the general radiation of long wave length which constitutes the major output of a Bucky tube is poorly adapted to calculations of the mechanism by which cells are killed. It should be noted that the experiments of this group with *B. coli* and with *B. aertryke* were made under sufficiently different conditions so that no significance attaches to the relative killing rates found.

No commercially available electron-type X-ray tube supplies a monochromatic beam intense enough for experiments on the killing rates of bacteria. The self-rectifying gas tube developed in this laboratory and described² elsewhere is, however, suitable for this purpose and, equipped with a copper target, has been employed in a second series of experiments with *B. coli* and *B. aertryke*.



TEXT-FIG. 1. A plot of the survival ratios resulting from the use of soft general radiation from a tungsten target X-ray tube (Table I).

In some instances the radiation from this tube has been filtered only by the 0.001 inch aluminum forming its window; in others a thickness of 0.0009 inch metallic nickel foil was interposed between the bacteria and the target. This filter cuts down the general radiation and eliminates 99 per cent of the K- β line at the same time passing half of the K- α doublet. The tube was operated at about 4 MA and 34 KV peak (unrectified wave). During a single series of experiments the variations in the current through the tube were less than 0.1 MA and its output of X-rays was found to be steady to within 1 per cent.

² Wyckoff, R. W. G., and Lagsdin, J. B., *Radiology*, July, 1930, 15, 42.

Each series required the exposure of about 100 plates, the results of which were averaged to give the survival ratios of Tables II and III. As Text-figs. 2 and 3 indicate, these ratios fall upon straight lines when plotted on semilogarithmic paper. Such a result agrees with the

TABLE II
Survival Ratios with Unfiltered Copper Radiation

Time	Survival ratios			
	<i>B. coli</i> (1)	<i>B. coli</i> (2)	<i>B. coli</i> (3)	<i>B. aertryke</i>
5 sec.	—	0.749	—	—
10 "	—	.617	0.645	0.793
20 "	0.358	.329	.548	.583
30 "	.233	.206	.422	.387
40 "	.167	.154	.344	.349
50 "	.112	—	.265	.270
60 "	.100	.089	.193	.234
Ionization current/sec./cm. ³	—	—	144.2 e.s.u.	—

TABLE III
Survival Ratios with Filtered Copper Radiation

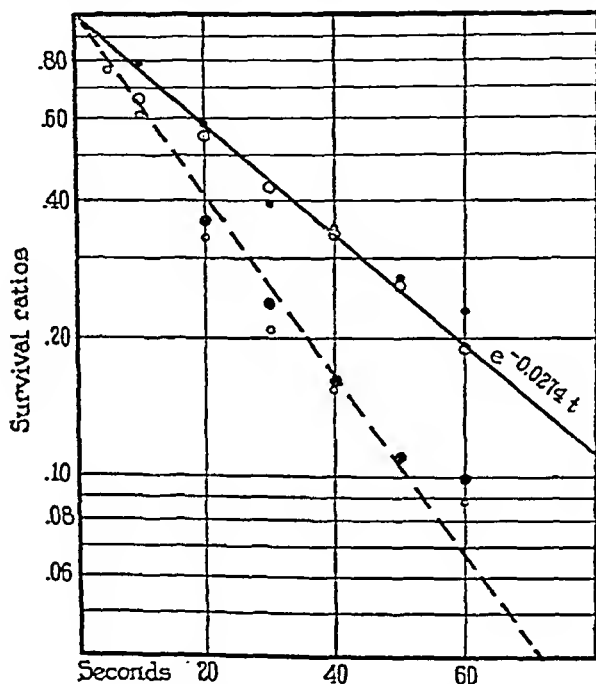
Time	Survival ratios		
	<i>B. coli</i> (1)	<i>B. coli</i> (2)	<i>B. aertryke</i>
20 sec.	0.667	0.711	0.804
40 "	.367	.574	.523
60 "	.345	.362	.391
90 "	.182	.291	.211
120 "	.109	.212	.189
Ionization current/sec./cm. ³	—	67.2 e.s.u.	67.2 e.s.u.

experiments of Holweck³ and Lacassagne³ on *B. pyocyaneus* with silver L-radiation.

The experimental arrangement is shown in Fig. 1. During use the stamping device for the standard area A and the irradiated area B replaced the diaphragm

³ Holweck, F., *Compt. rend.*, 1929, 188, 197; Lacassagne, A., *Compt. rend.*, 1929, 188, 200.

C under the X-ray tube D. When set up, the tube D was carefully centered with the aid of a fluorescent screen to insure that the X-ray beam covering B should be uniform. A Petri dish carrying the desired number of bacteria spread upon its agar surface was placed on the stand E and raised by the remote control rod F until the knife edges of A and B cut through the agar. On opening the metal shutter G, the surface marked by B could then be given an exposure of desired length. After lowering E, it and the dish were removed with the help of F. This remote control was needed because the X-ray tube itself operated continuously

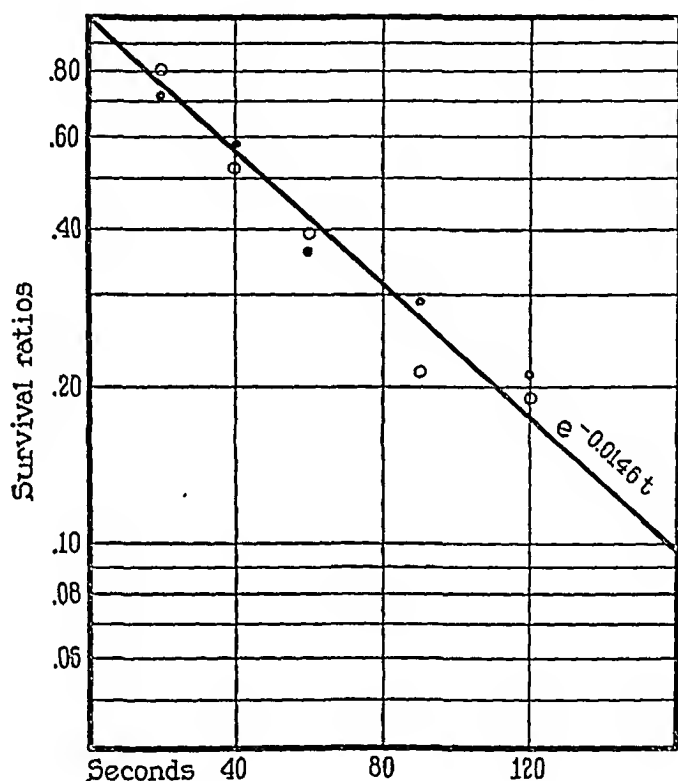


TEXT-FIG. 2. A plot of the data obtained by irradiating *B. coli* and *B. aertryke* with filtered copper rays. The small open, large black, large open and small black circles refer to experiments (1), (2), (3) and (4) of Table II.

throughout the entire experiment. When in use, the tube was covered by a lead housing such as that shown at H and during exposures the door K was kept closed. These precautions are essential to the safety of the experimenter.

The intensity of the X-rays striking the bacteria spread on the agar surface was obtained from measurements of the amount of ionization in air produced by these rays. Because of their absorbability, it was

considered inaccurate to employ for this purpose a small enclosed type ionization chamber. Accordingly, an open air chamber of standard design but of small size was made. Its over-all length was 7.0 cm. The actual length of the amber-insulated collecting electrode was 1.31 cm., its effective length 1.39 cm. The grounded electrodes on either side were each 2.54 cm. long. All three electrodes were 3.8 cm. wide. The charged shield surrounding and facing these plates was 3.2 cm.



TEXT-FIG. 3. A plot of data obtained by irradiating *B. coli* and *B. aertryke* with the same dose of unfiltered copper rays. The small black and large open circles refer to experiments (2) and (3) of Table III.

distant. On account of the extremely great ionizing power of the X-rays used, saturation in a chamber of this type can be obtained only if beams of small cross-section are measured. The currents produced in this chamber were measured by the usual balance methods⁴ employing

⁴ See for instance, Makower, W., and Geiger, H., *Practical Measurements in Radio-activity*, London, 1912, 15.

a variable voltage and a standard condenser of known capacity. This capacity was obtained by comparison against a small standard condenser and checked by calculation from its accurately determined dimensions.

The intensity of the X-rays striking a small area of the agar surface was found by replacing the stamping device AB of Fig. 1 with a small lead stop, C, having an opening 0.117 cm. in diameter. The air chamber was brought as near to the stop as possible (ca 3 mm.) and the ionization produced by the X-ray beam passing through it was ascertained. Knowing the length of the measured column of ionized air and its absorption coefficient under the conditions of temperature and pressure of the experiment and for copper rays, it is obviously possible to calculate the approximate ionizing power of the beam at the irradiated surface. A typical calculation will make clear the procedure followed and the approximations introduced.

Area of stop in position of bacterium = 0.0107 cm.²

Height of air column the ionization of which is measured = 1.39 cm.

Corrected density of air = 0.001165

Temperature = 26°C., Barometer = 747 mm. Hg.

Absorption coefficient μ/ρ of air for copper radiation = 8.43

Balancing voltage on potentiometer = 18.8 V in 20 sec.

1 c.s.u. potential = 0.33×10^{-2} volts

Measured capacity of standard condenser = 321.4 cm.

Therefore the observed ionization current is

$$i' = \frac{18.8 \times 0.33 \times 10^{-2} \times 321.4}{20 \text{ sec.}} = 0.997 \text{ c.s.u.}$$

Since $I/I_0 = e^{-\mu l}$ where l is the length in centimeters of the absorbing column and $\mu = 8.43 \times 0.001165$, a column of air 1 cm. long will absorb 0.721 as much as a similar column of length 1.39 cm. Hence the ionization produced per second in 1 cm. depth of air by the rays striking 1 cm.² of irradiated bacterial surface is taken as

$$i = \frac{i' \times 0.721}{0.0107} = 67.2 \text{ c.s.u. per cm.}^2 \text{ per sec.}$$

The intensities of the X-rays used in each of the three final standardized experiments when expressed in the foregoing units are recorded in Tables II and III.

Analysis

Existing knowledge of the properties of X-rays and of the mechanism of their absorption in inorganic matter makes it seem inevitable

that their absorption in bacteria is a quantized process. Thus the X-rays incident upon a cell will either pass through without altering it or else they will give up one or more quanta whose energy content is connected with the wave length λ of the rays through the familiar relation

$$E = h \nu = h \frac{\lambda}{c}$$

where h is Planck's constant, ν is the frequency of the rays and c is the velocity of light. It is known that a high velocity electron is liberated as a result of such an absorption. This electron gives rise to a chain of ions in the matter through which it passes and to X-rays which, in their turn, liberate more ions of less and less energy. The volume within which this cluster of ions resulting from a single quantum absorption is freed increases rapidly with the magnitude of the original quantum but, in matter having the density of a bacterium, it is at greatest only a very small fraction of a cubic millimeter. The changes X-rays produce in protoplasm are naturally identified with the physico-chemical changes induced by this ionic shower.

The physical consequences of the absorption of an X-ray quantum so closely resemble those attending the direct absorption of a high velocity electron that it seems permissible to treat the killing action of X-rays by the same statistical analysis which has previously been used in studies of the killing of bacteria by cathode rays.

On this basis, the straight line obtained by plotting survival ratios upon semilogarithmic paper means that, on the average, the death of a bacterium is the result of the absorption of a single X-ray quantum. As the earlier analysis⁵ has shown, survival ratios under these conditions can be calculated from the expression

$$\frac{A_1}{A_0} = e^{-\alpha t}$$

where t is the time and α is the expected, or average, number of quantum absorptions in unit time.

A more intimate picture of the killing process is to be had from a study of this expected number of hits. Under conditions of satura-

⁵ Wyckoff, R. W. G., and Rivers, T. M., *op. cit.*

tion, the air ionization chamber measures the total number of ions produced by the quanta absorbed in a known volume of air. If the number of pairs of ions liberated by a single quantum can be judged, then the number of quanta absorbed in this volume of air is known. Furthermore, if the ratio of the absorption coefficients of air and of a bacterium can be estimated, the *average* number of quanta absorbed by a single bacterium in unit time is readily computed from the size and shape of the organism. This has been done for experiments with filtered and unfiltered copper rays.

The following typical calculations based upon the standardized experiment of Table III using *B. aertryke* will show how the average number of hits has been obtained and will make clear the approximations that have been introduced in the process.

$$67.2 \text{ e.s.u.} \approx 67.2 \times 0.21 \times 10^{10} \text{ ion pairs/sec.} = 1.411 \times 10^{11} \text{ ion pairs/sec./cm.}^3$$

A small amount of radiation is absorbed in the air between the diaphragm C and the collecting electrode of the air chamber. This absorption is readily calculated from the dimensions of the apparatus and the absorption coefficient ($\mu/\rho = 8.43$) of copper radiation. With the resulting correction it is found that 1.46×10^{11} ion pairs will be produced per second per cm.^3 at the position of the irradiated bacteria. In the absence of direct experimental knowledge of the absorption coefficient of protoplasm, the mass coefficients of air and of living cells are customarily assumed equal. If this assumption, which cannot depart far from the truth, is made, the amount of absorption in 1 cm. thickness of air is about 27.9 times that of one bacterium. Both the *B. coli* and the *B. aertryke* have been taken as rods 0.5μ in diameter and 2μ long. Computation shows that to a first approximation one bacillus may be considered equivalent in absorption to a rectangular block of protoplasm 1×10^{-8} cm. in area, 0.42μ thick and of unit density. From these quantities the average number of ion pairs absorbed per bacterium per second becomes

$$\frac{1.46 \times 10^{11} \times 1 \times 10^{-8}}{27.9} = 52.3 \text{ ion pairs/sec./bacterium.}$$

In order to obtain the number of X-ray quanta absorbed per second it is necessary to know how many ions are liberated when one quantum is absorbed in air. The best available measurements⁵ indicate that for X-rays of the quality used in these experiments about 35 volts are required to produce each electron pair. The voltage equivalent of the K- α lines of copper as computed from the familiar quantum relation

⁵ Kulenkampff, H., *Ann. d. Physik*, 1926, 79, 97.

$$\text{Voltage (in KV)} = 12.34/\lambda(\text{in \AA}) = 12.34/1.537$$

is 8.029 KV. From this the number of ion pairs arising through the absorption of one quantum of Cu K- α radiation is

$$8029/35 = 229.$$

The average number, α , of quantum absorptions per second then is

$$52.3/229 = 0.228.$$

The observed survival ratios in Table III lead to a straight line having the equation $A_1/A_0 = e^{-0.0146t}$ (Text-fig. 2). It will be noted that this experimental $\alpha' = 0.0146$ is much smaller than the α calculated from ionization measurements.

DISCUSSION

Accepting the foregoing type of analysis as an essentially correct, if rough, description of what happens when X-rays strike bacteria, the smallness of the ratio α'/α when taken in connection with the linearly exponential character of the experimental results shows that though on the average the absorption of one quantum of these radiations is sufficient to kill a bacterium of either *B. coli* or *B. aertryke*, relatively few of the absorbed quanta are lethal. In the experiment just calculated 0.0146/0.228 or about one in 15.6 kills. In the standardized experiment of Table II using *B. coli* one in 17.8 is deadly ($\alpha = 0.489$, $\alpha' = 0.0274$). This agreement is satisfactory since it is scarcely to be expected that the results with filtered and unfiltered rays should be identical.

The fact that so many quanta can be absorbed by a bacterium without causing death apparently means that the vital elements within the cell which can be destroyed by a direct quantum hit are much smaller than the cell itself. If the volume through which the quantum acts were negligible compared to that of the vital element of the cell and if there were only one such element in a bacterium, then the "sensitive volume" that would have to be hit in order to bring about death would be α'/α . In the two standardized experiments with filtered rays this ratio is 0.064; in the series using unfiltered radiation it is 0.056. The spheres of action of these quanta cannot, however, be disregarded and the quantities α'/α are to an important degree measures of these regions of quantum action. Furthermore, if, as seems natural, the

vital parts of the cell are identified with its chromatin material, more than one "sensitive volume" exists within it. It can nevertheless be concluded that the volume of the "vital elements" contained within one of the bacteria studied scarcely exceeds 0.05 of its entire volume. This estimate seems to be in serious conflict with the earlier conclusions of Holweck and Lacassagne with *B. pyocyaneus*. The published data are, however, too few to allow of a satisfactory comparison.

Within the limits of experimental error there is no difference between the sensitiveness of *B. aertryke* and *B. coli* to copper K-radiation (Text-fig. 2). A similar result was found when studying the killing action of cathode rays upon them.

Additional experiments are being carried out to ascertain in what way differences in X-ray wave length affect the killing of these organisms.

Valuable help in the carrying out of these experiments has been given by Charles G. Porskieves.

CONCLUSIONS

Both copper K X-rays and the soft general radiation from a tungsten tube operated at 12 KV kill *B. coli* and *B. aertryke* in a linearly exponential fashion. Within the experimental limits, the two organisms appear to be equally sensitive to these radiations.

By making use of the fact that X-ray energy is absorbed in quanta, an approximate picture can be formed of the mechanism of this destructive action. If the average numbers of quanta (α) absorbed per bacterium per second are calculated from measurements of air ionization using the quantities outlined in the text, survival ratios for these bacilli can be approximately represented by the equations

$$\frac{A_1}{A_0} = e^{-0.064 \alpha t}$$

for filtered copper rays and

$$\frac{A_1}{A_0} = e^{-0.056 \alpha t}$$

for unfiltered copper rays (peak voltage = 34 KV).

In terms of the foregoing interpretation this means that when death results, it is caused by the absorption of a single X-ray quantum of energy. Since only about one in twenty of the absorbed quanta kills, the sensitive cell constituents whose destruction leads to cell death must have a volume which is less than 0.06 of the bacterium itself.

EXPLANATION OF PLATE 17

FIG. 1. A photograph of the experimental arrangement for irradiating bacteria.

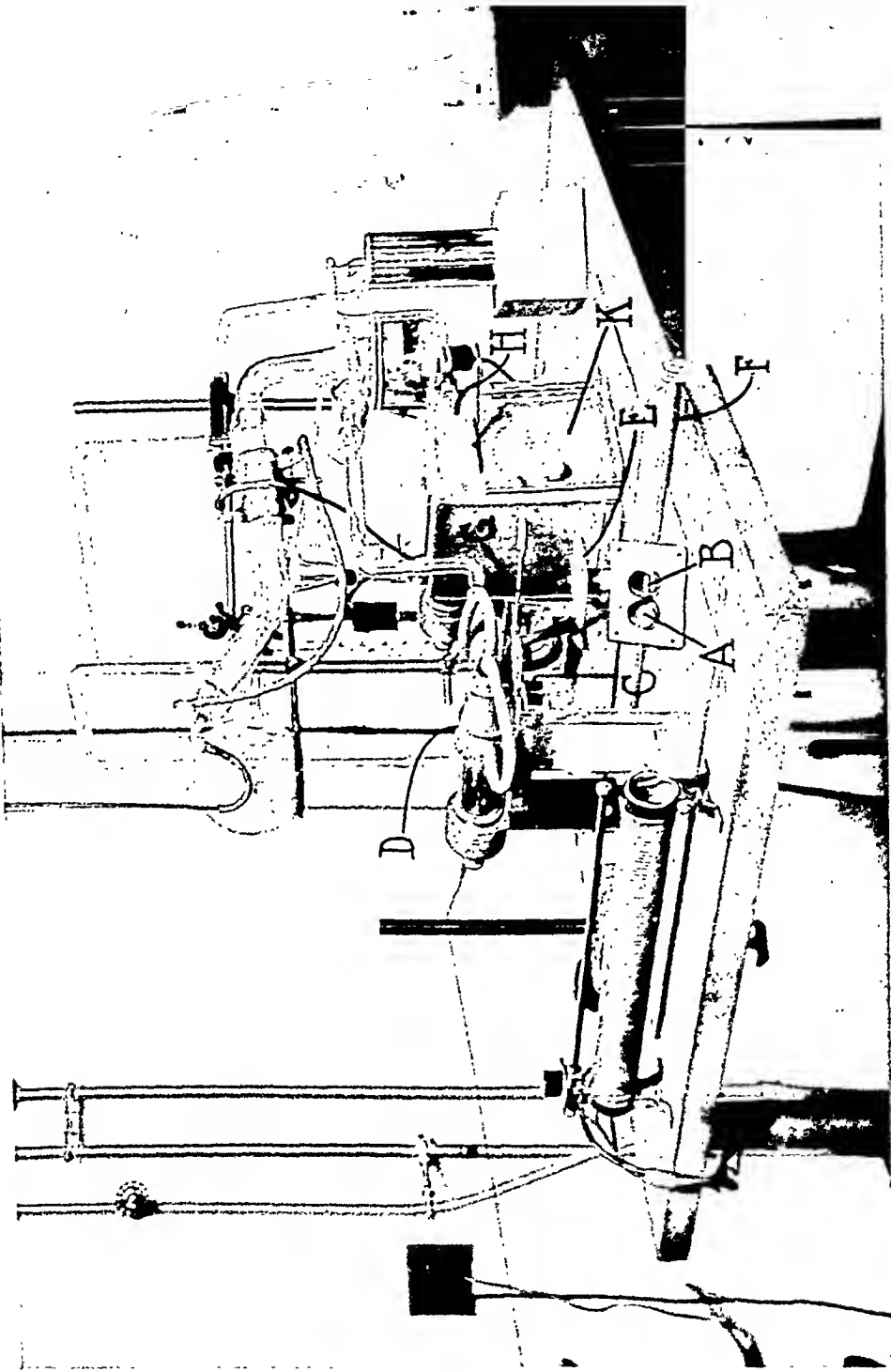


Fig. 1

(Wyckoff: Filling of certain bacteria by Nitrogen)

THE CATALYTIC EFFECT OF METHYLENE BLUE ON THE OXYGEN CONSUMPTION OF TUMORS AND NORMAL TISSUES

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It has previously been shown (1, 2, 3) that the addition of methylene blue, or similar reversibly oxidized and reduced dyes, increases the oxygen consumption of cell suspensions. It was also demonstrated that correlated with this increase, there is a decrease in the aerobic glycolysis* as shown by a diminished lactic acid formation (4). It was furthermore suggested (5) that the effect of the dye seemed to be proportional to the fermentative power of the cell. To test the validity of this hypothesis, use has been made of the fundamental researches of Warburg (6) on the metabolism of normal tissues and tumors. Warburg has shown there is a sharp difference between the metabolism of tumors and that of normal adult tissues. While in general normal adult tissues possess a high respiration, sufficient to check the appearance of the fermentative processes, tumors, on the other hand, have a low respiration, and consequently part of the energy necessary for the performance of cellular activities has to be provided by fermentation. The lactic acid produced in tumors may thus reach about 13 per cent of the weight of the tumor. If methylene blue and similar dyes act as catalysts for the oxidative processes of living cells only when the cells in question possess a fermentative power, these two kinds of tissues are the most appropriate material with which to test the validity of the hypothesis.

The experiments were performed on fresh rat tissues and rat tumors taken soon after death caused by a blow on the head. The tissues and tumors were cut with

* Whenever the word glycolysis is employed in this paper, it is used in the sense given by Warburg, *i.e.*, the splitting of one molecule of glucose into two molecules of lactic acid according to the equation $C_6H_{12}O_6 = 2C_3H_7O_2$.

a hand razor into thin slices, 0.4 mm. thick, to allow free diffusion of oxygen throughout the section. They were kept in a saline solution containing 0.15 gm. per cent of glucose, and a mixture of phosphate buffers, $\frac{M}{15}$ having a pH of 7.38. The oxygen consumption was determined in Warburg vessels with Barcroft manometers. The temperature of the water bath was 37.5°C.

The Effect of Methylene Blue on the Oxygen Consumption of Normal Tissues

The following normal tissues were employed: liver, kidney, brain (gray matter), spleen, and testicles. For the study of pancreas, rabbits were used. The respiration and carbohydrate metabolism of slices of these tissues have been determined *in vitro* by Warburg and his associates. Liver, pancreas and kidney possess no aerobic glycolysis. Brain and testicles are reported as having a small aerobic glycolysis. The metabolism of spleen has been determined by Murphy and Hawkins (7) and found to show an aerobic glycolysis.

In preliminary determinations, the metabolism of all these tissues was measured by the use of the well known Warburg methods, and found to be more or less like that reported by the above named investigators, except for the metabolism of rat testicles, where a QO_2

of -15.0 was found, and a QCO_2 of $+1.2$ instead of $QO_2 - 12.3$ and $QCO_2 + 7.2$

found by Warburg. In view of this discrepancy, the glucose consumption and lactic acid formation of rat testicles were determined chemically. As it can be seen from Table 1 there was no lactic acid formation, though there was destruction of glucose. The addition of methylene blue did not have any influence either on the speed of glucose consumption or in the lactic acid formation.

TABLE 1

The Effect of Methylene Blue on the Glucose Consumption and Lactic Acid Formation of Rat Testicles

Testicles continuously shaken in a saline solution containing phosphate buffer $\frac{M}{15}$ pH 7.38.

437.8 mg. testicle	Glucose in mg. per cent	Lactic acid in mg. per cent
Before incubation	114.0	66.9
2 hours after incubation control	87.0	66.4
M. B. added	87.0	66.8

TABLE 2

The Effect of Methylene Blue on the Oxygen Consumption of Normal Tissues

Kind of tissue	O ₂ consumption in c.mm. per hour		Per cent increase or decrease
	Before dye addition	After dye addition	
Rat kidney:			
1).....	47.6	47.3	-0
2).....	128.8	116.3	-9.7
3).....	59.0	56.2	-4.7
4).....	51.6	38.0	-26.3
5).....	63.0	46.5	-26.2
6).....	94.0	79.0	-15.9
Rat liver:			
1).....	37.2	37.5	-0
2).....	80.6	75.0	-6.9
3).....	41.7	40.3	-3.3
4).....	42.7	37.4	-12.4
5).....	54.2	52.2	-3.7
Rabbit pancreas:			
1).....	25.6	23.4	-8.6
2).....	22.0	20.6	-6.8
3).....	17.5	13.0	-25.7
4).....	23.6	19.0	-19.5
5).....	24.8	22.6	-8.9
Rat testicle:			
1).....	120.5	119.5	-0
2).....	118.9	114.9	-3.4
3).....	117.2	114.0	-2.7
4).....	140.7	140.0	-0
Rat spleen:			
1).....	31.0	33.4	+7.7
2).....	29.7	30.8	+3.7
3).....	38.3	42.1	+9.9
4).....	32.0	34.2	+6.9
5).....	34.8	37.0	+6.3
6).....	55.1	55.4	0
7).....	46.8	57.4	+22.7
Rat brain (gray matter):			
1).....	28.6	30.3	+6.0
2).....	35.3	38.6	+9.3
3).....	37.2	37.6	0
4).....	33.0	41.8	+26.6
5).....	25.8	31.9	+23.6
6).....	28.8	33.2	+15.3
7).....	29.2	31.7	+8.5

In Table 2 have been tabulated the results of the experiments on the oxygen consumption of normal tissues, and the effect of methylene blue. The values given do not refer to the weight of the tissue, but are the figures directly found. The oxygen consumption of these tissues, weighed after drying for some hours at 100°C., was found to correspond to the figures given by Warburg. In the tissues where there is no aerobic glycolysis, *i.e.*, lactic acid production, methylene blue has no catalytic effect; moreover, it exerts a definite toxic action as shown by a definite drop in the oxygen consumption. Methylene blue increases the oxygen consumption of brain tissue and spleen,



which possess an aerobic glycolysis of $QCO_2 + 2.5$ and $+2.3$ respectively. Methylene blue does not increase the oxygen consumption of the tissue of rat testicles, nor does it have action on the lactic acid formation. It is interesting to note that the toxic effect of the dye observed in kidney and liver tissues is not found in testicles.

In order to provide further evidence of the action of methylene blue on the fermentation phase of cellular metabolism, the oxygen consumption of the above mentioned tissues was measured in the presence of KCN ($\frac{M}{3000}$), previously neutralized to the same pH as that of the saline solution, and the effect of methylene blue determined afterwards. Cyanide is a specific respiratory poison as shown by Warburg, but it has no effect on the fermentative process which proceeds undisturbed. Incidentally, Dixon and Elliot's (8) observations on the influence of cyanides to inhibit partially the power of normal tissues to consume oxygen were confirmed.* While KCN inhibits completely the respiration of goose erythrocytes, yeast cells and *Arbacia* and starfish eggs, this inhibition is never complete in the case of normal tissues. In Table 3 are given average figures of the experiments. Liver, kidney, pancreas, and testicles, which normally did not show any increase in their oxygen consumption by the action of methylene blue, exhibit a manifest increase when the respiration is inhibited by cyanide and the glycolysis appears. In spleen, the increase of the oxygen

* After this paper had been sent to press, Howard, from Warburg's laboratory, showed that KCN inhibition of respiration is about complete when the tissues are kept in Ringer solution buffered with bicarbonate (*Biochem. Z.*, 1930, 221, 498). This recent communication does not affect the experiments related here.

consumption by the action of methylene blue is considerably enhanced by previous addition of cyanide.

Wendel (9) has reported that red blood corpuscles oxidize added sodium lactate in the presence of methylene blue. The addition of lactates to normal tissues, where the catalytic power of the dye is absent, produces neither an increase on the oxygen consumption nor a diminution of the added lactates. The determinations were made with liver, kidney and testicles, both by the manometric methods and by chemical analysis.

TABLE 3

The Effect of Methylene Blue on the Oxygen Consumption of Normal Tissues Previously Treated with KCN ($\frac{M}{1000}$)

Kind of tissue	O ₂ consumption per hour in c.mm.		Per cent increase
	Before dye addition	After dye addition	
Rat kidney.....	22.9	36.3	58.5
Rat liver	13.8	16.4	18.8
Rat spleen	5.2	20.1	286.4
Rat testicle	9.2	27.6	200.0

The Effect of Methylene Blue on the Oxygen Consumption of Tumors

As a result of the observations of Warburg, repeated and confirmed by a number of investigators, it is known that tumor tissues possess a metabolism different from the metabolism of normal adult tissues. Tumors, whether taken from animals or cultivated *in vitro* show in general a low respiration compensated by an abnormally high fermentation, which is more manifest when measuring the "Pasteur Reaction" of these tissues, *i.e.*, the excess fermentation given by the following formula $= U = Q_M^{\text{ana}} - 2 (QO_2)$ where Q_M^{ana} is the anaerobic glycolysis and QO_2 the respiration. Let us add that aerobic glycolysis is not a specific feature of tumor tissues. Warburg (10) has summarized examples of normal tissues possessing aerobic glycolysis. Crabtree (11) has shown it to be a property of certain pathological overgrowths associated with intracellular viruses, and recently Neuhaus (12) has reported the presence of an appreciable aerobic glycolysis in granulation tissues.

TABLE 4

The Effect of Methylene Blue on the Oxygen Consumption of Tumors

Kind of tumor	O ₂ consumption in c.mm. per hour		Per cent increase
	Before dye addition	After dye addition	
Human carcinoma (breast):			
1).....	17.0	21.3	25.3
2).....	24.6	33.7	37.0
3).....	26.0	31.0	19.2
Rat carcinoma (Walker 256):			
1).....	24.4	40.5	66.0
2).....	22.5	29.5	31.1
3).....	32.4	40.3	24.4
4).....	29.2	42.3	44.8
5).....	16.3	33.6	106.0
6).....	24.3	38.4	58.0
7).....	25.1	36.5	45.4
8).....	32.0	48.0	50.0
9).....	12.7	21.9	72.4
10).....	28.1	46.3	64.8
11).....	32.3	48.5	50.2
Rat adenocarcinoma No. 20:			
1).....	14.3	25.6	79.0
2).....	25.9	45.4	75.2
3).....	19.7	37.3	89.3
Rat sarcoma No. 1 (Walker):			
1).....	24.1	33.3	38.2
2).....	26.6	40.3	51.5
3).....	32.9	48.8	48.3
4).....	35.3	47.4	34.3
5).....	49.7	62.8	26.4
Rat sarcoma No. 135:			
1).....	38.7	57.0	47.3
2).....	30.5	42.5	39.3
3).....	41.3	54.5	32.0
Rat sarcoma No. 10:			
1).....	29.9	41.0	37.1
2).....	34.4	53.9	56.7
Chicken sarcoma (Rous):			
1).....	11.6	23.5	102.0
2).....	10.5	22.7	116.0
3).....	9.4	19.6	108.6
4).....	17.4	27.0	55.2
5).....	10.1	14.2	39.4

It was expected that methylene blue would exert its catalytic power in the presence of tumors, since they possess a high aerobic glycolysis. Table 4 demonstrates that such is the case. Different kinds of tumors were used in these experiments: human carcinoma, rat sarcoma, rat adenocarcinoma, and Rous chicken sarcoma with the same results, namely that there is a definite increase in the oxygen consumption of these tissues in the presence of methylene blue, an increase which is higher than the one observed in brain and spleen.

When the respiration is inhibited by cyanides, the addition of methylene blue, although increasing considerably the oxygen consumption in relation to the previous low value obtained before the

TABLE 5

The Effect of Methylene Blue on the Oxygen Consumption of Tumors, Previously Treated with KCN ($\frac{V}{V_0}$)

Kind of tumor	O ₂ consumption per hour in c.mm.		Per cent increase
	Before dye addition	After dye addition	
Human carcinoma	1.5	7.1	373.4
Rat adenocarcinoma (Walker No. 76)	0	15.0	∞
Rat carcinoma, mammary gland (Walker No. 256)	0.5	7.7	1440.0
Rat sarcoma (Walker No. 1)	3.8	16.5	337.0

dye is added, does not bring it back to the values observed without cyanides. There is in the action of cyanides on the oxygen consumption of tumors an interesting phenomenon which will be studied more carefully in this laboratory. KCN inhibits the oxygen consumption of tumors almost completely. In the experiments here reported the inhibition was from 100 per cent to 90 per cent in relation to the oxygen consumption of the same weight of untreated tumor. This observation is in sharp contrast to the findings of Dixon and Elliot (8), which have been confirmed in this laboratory, of the incomplete inhibition of the oxygen consumption of normal tissues by cyanides, which, in the experiments reported in Table 3, was from 70 to 85 per cent.

The Relation between the Catalytic Power of Methylene Blue and the Aerobic Glycolysis of Cells and Tissues

In an attempt to correlate, in a quantitative way, the relation between the catalytic power of methylene blue, and the fermentative power of the tumors studied, the aerobic glycolysis was determined, employing the methods of Warburg. The data have been tabulated in Table 6. The figures given for the aerobic glycolysis of normal tissues have been taken from Warburg's data (13), excepting that the aerobic glycolysis of spleen has been taken from the data of Murphy and

TABLE 6

Relation between the Aerobic Glycolysis, and the Catalytic Power of Methylene Blue on the O₂ Consumption of Tumors and Normal Tissues

Kind of tumor or tissue	Per cent increase or decrease due to M. B. (O ₂ consumption)	Aerobic glycolysis
Kidney.....	-13.8	0
Liver.....	-5.2	0
Pancreas.....	-13.9	0
Spleen.....	+8.2	+2.3
Brain.....	+12.7	+2.5
Human carcinoma.....	+27.2	+9.2
Rat carcinoma (Walker).....	+55.7	+17.7
Rat adenocarcinoma (Walker).....	+81.7	+22.5
Rat sarcoma (Walker No. 1).....	+39.7	+11.3
Rat sarcoma (Walker No. 135).....	+39.5	+12.1
Chicken sarcoma (Rous).....	+84.2	+21.4

Hawkins. The data on the aerobic glycolysis of Rous chicken sarcoma have also been taken from the last mentioned authors. It can be seen in this table that methylene blue exerts its catalytic power only on cells or tissues possessing aerobic glycolysis. There is evidently a rough proportionality between the catalytic power of the dye and the fermentative power of the cell or tissue.

DISCUSSION

It has been established (5) that the following factors influence the catalytic power of reversibly oxidized and reduced dyes on the cellular oxidative processes: the permeability of the cell membrane to the

dye; and the reduction potential of the dye, since the speed of this activation is a function of the speed at which the dye is reduced by the cell, and the speed at which the leucodye is oxidized by the atmospheric oxygen. From the experiments reported in this paper it can be said that the fundamental factor is the fermentative power of the cell or tissue. Thus normal adult tissues have, in so far as the microscopic sections can reveal, the same permeability for methylene blue as tumors; they possess the same reducing power as shown by Voegtlin, Johnson and Dyer (14). Nevertheless, while the dye has no action or even exerts a toxic effect on the respiration of normal adult tissues when deprived of aerobic glycolysis, the dyes show their catalytic power on cells or tissues possessing a fermentative power as expressed by their aerobic glycolysis. Methylene blue has no effect on the oxygen consumption of most normal adult tissues because they do not produce lactic acid under aerobic conditions. The high respiration of the cell checks the fermentative power. When the respiration of these tissues is inhibited, by the addition of a specific respiratory poison, namely KCN, and the fermentation appears, methylene blue exhibits its catalytic power, increasing the oxygen consumption of these tissues, normally inactive to the action of the dye.

The effect of methylene blue on the oxygen consumption of tumors is a further confirmation of this point of view. It is known that the respiratory power of the tumors is limited; as a consequence, and, according to the brilliant conceptions of Pasteur (15), because respiration is performed at the expense of fermentation, the fermentative process in aerobic conditions appears to provide the energy necessary for the maintenance of the cellular metabolism. Neoplasms therefore provide the necessary conditions for the action of methylene blue. An extremely interesting question is raised here, a problem which will be investigated shortly. If it is possible to change the metabolism of tumor tissues by the addition of methylene blue in such a way that the tissue shows an increased respiration, as well as a decreased aerobic glycolysis with a consequent shift of the Pasteur Reaction towards the negative side, it means, that, by the action of the dye, the metabolism of these tumors is shifted towards the direction of the metabolism of normal adult tissues. By using a small concentration of methylene blue on cultures of tumors, and growing them for several generations in a medium containing the dye, possibly the characteristic

tumor metabolism, with its low respiration and high fermentation, might be changed permanently into one showing the characteristics of normal tissues.

The constant relation between the fermentative power of cells and tissues, and the increase of their oxygen consumption after the addition of methylene blue is so general and has been tested with such a variety of cells and tissues, that it seems justifiable to suggest the use of methylene blue as a test for the fermentative power of cells and tissues.

CONCLUSIONS

1. Methylene blue has no catalytic effect on the oxygen consumption of those normal adult tissues which do not possess aerobic glycolysis. The dye increases the oxygen consumption of these tissues when their respiration has been inhibited by the addition of KCN and their fermentative power thus brought into action.

2. Methylene blue increases the oxygen consumption of normal tissues having aerobic glycolysis, and of tumors.

3. The effect of methylene blue is roughly proportional to the fermentative power of tissues.

I wish to express my sincere thanks to Dr. Margaret Reed Lewis of the Carnegie Laboratory of Embryology for generously supplying me with the rat tumors used in these experiments.

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IMMUNITY TO POLIOMYELITIS IN MOTHERS AND THE NEWBORN AS SHOWN BY THE NEUTRALIZATION TEST*

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Evidence has been presented elsewhere relating to the age distribution of poliomyelitis which indicates that immunity to the disease is widespread, bearing a direct relationship to age and concentration of population and an inverse relationship to the incidence of the disease in all age groups.† It was inferred from this study that the virus of the disease has a similar widespread distribution (1). Neutralization tests on a series of individuals not giving a history of poliomyelitis were reported in a later communication (2). In an urban population im-

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† While increasing immunity with increase in age is undoubtedly a major factor in determining the diminishing incidence of the disease with increase in age, there are indications that especially after adult age is reached a diminishing chance of adequate exposure is also a factor not only in the diminishing incidence of the disease but also in a diminution in the rate with which immunization occurs with increase in age. Diminishing chances of contact infection with age are also suggested by Chapin's figures for measles, which show a diminishing attack rate in susceptibles in families with cases with increase in age (5), as well as by Pope's studies of scarlet fever (6). The idea of diminishing chance of exposure with increase in age is also in line with certain indications that in adults there tends to be a loss of immunity which might be accounted for by lack of reinforcement from repeated exposures. For rural populations both our own neutralization tests for immunity to poliomyelitis and Kidder's figures for immunity to diphtheria (7) indicate a slightly higher susceptibility amongst adults in general than for the 15 to 19 year age group.

munity was found to exist least often in the 1 to 5 year age group, but with increasing frequency with increase in age. In a rural population not only was the frequency of immunity in the 1 to 5 year age group less but the increase in the frequency with which it was found with increase in age was less than in the urban population. Evidence has also been presented that urban adults residing in a warm climate enjoy a degree of immunity to the disease similar to that of urban adults living in a cool climate (3). This suggests a similar widespread distribution of the virus in warm and cool climates, and in view of the greatly diminished incidence of the disease in warm climates, emphasizes the point brought out elsewhere that the occurrence of the disease may not in all circumstances parallel the distribution of the virus (4).

The results of these studies of the extent of immunity in relation to the incidence of the disease with respect to age and concentration of population when compared with similar studies on diphtheria suggest that the mechanism of dissemination of the virus and of immunization is the same as in diphtheria, the essential difference in the epidemiology of the two diseases lying in the frequency with which the "accident" of disease occurs in the process of immunization.

In infants under 1 year of age the incidence of both poliomyelitis and diphtheria is low. In the case of diphtheria the relatively low incidence in the first year of life has been shown to be due to an immunity passively derived from the mother. This passive immunity disappears in most instances before the age of 1 year is reached. Polano (8) in 1904 in testing the blood of seven mothers and the cord blood from their infants found that the amount of antitoxin was quantitatively similar or was absent in each. Von Groer and Kassowitz (8) testing mothers and infants at 143 births found antitoxin in 84 per cent of infants. In 96 per cent of these, mothers and infants corresponded with one another in having or not having antitoxin. Zingher, (8) in 1917, working with the Schick test upon 23 mothers and infants up to 3 months of age, found only a single divergent result, namely, a positive test (no antitoxin) in an infant from a Schick negative mother.

In order to determine whether the relatively low incidence of poliomyelitis in infants is similarly due to temporary maternal passive immunity we have tested the blood sera of a group of mothers together with sera from the cord blood of their newborn infants for the presence of neutralizing substance for the virus of poliomyelitis. The

sera were mixed with a virus suspension, incubated for 2 hours, placed in the ice box over night, and inoculated intracerebrally into *rhesus* monkeys in the manner described in our earlier papers (2, 3). The results of these tests are shown in Table I.

The serum of nine mothers and their infants completely neutralized the virus. The serum of one infant neutralized the virus while the animal inoculated with the mixture of virus and the mother's serum developed the disease on the seventeenth day following inoculation. The control animal in the same experiment developed the disease on the eighth day.* The serum of two mothers and their infants failed to neutralize the virus.

These results may be taken as an indication that where the mother is immune to poliomyelitis there is a passive transmission of immunity to the infant. We have not performed any tests to determine the duration of this immunity or whether or not it is fortified or prolonged by suckling, as has been shown to be the case in diphtheria. However, previous tests showing immunity in a relatively low proportion of children in the 1 to 5 year age group together with the relatively high incidence of the disease in this age group suggest that the duration of passive immunity in infants is not unlike that in diphtheria.

DISCUSSION

In this small series of tests immunity was found in a correspondingly high percentage of infants at birth and their mothers, the infants corresponding in each instance with the mother in respect to immunity with one exception, in which the result was doubtful but not contradictory. The fact that even in this small series the findings agree with what was anticipated from an analysis of the age distribution of poliomyelitis in comparison with that of diphtheria justifies the conclusion that the relative infrequency of poliomyelitis under one year of age is due in considerable measure to a passive transmission of immune bodies to the young through the placenta. No tests have been done to determine whether this passive immunity receives reinforcement

* While the result of this test is doubtful, from our previous experiments we have come to regard an incubation period of more than 14 days (using an active 7 or 8 day virus) as probably representing partial neutralization, i.e., a degree of immunity.

TABLE I

Neutralization of the Virus of Poliomyelitis by Serum of Mothers and Newborn Infants

Case	Monkey	Virus No.	Virus-serum mixture 1:1	Date of inoculation	Outcome	Neutralization	Remarks
44780 (mother) 21 yrs.	49-8	515	cc. 1.0	4/ 8/30	No symptoms	+*	
44780 (baby)	48-9	515	1.0	4/ 8/30	No symptoms	+	
44798 (mother) 17 yrs.	47-6	515	1.0	4/ 8/30	No symptoms	+	
44798 (baby)	47-0	515	1.0	4/ 8/30	No symptoms	+	
44799 (mother) 19 yrs.	39-8	515	1.0	4/ 8/30	4/19 Paralyzed	-	Recovered with residual paralysis
44799 (baby)	49-5	515	1.0	4/ 8/30	4/21 Paralyzed	-	Typical microscopic lesions of poliomyelitis†
Convalescent serum No. 83	49-2	515	1.0	4/ 8/30	No symptoms	+	
Normal monkey serum	47-4	515	1.0	4/ 8/30	4/16 Paralyzed	-	Typical microscopic lesions of poliomyelitis
44803 (mother) 22 yrs.	49-9	515	1.2	4/10/30	4/17 Paralyzed	-	Typical microscopic lesions of poliomyelitis
44803 (baby)	34-5	515	1.2	4/10/30	4/18 Paralyzed	-	Typical microscopic lesions of poliomyelitis

* In the seventh column of the table *plus* indicates that the serum did neutralize the virus and *minus* that the serum failed to neutralize. *Plus* and *minus* indicates a doubtful or partial neutralization.

† Where *Typical microscopic lesions of poliomyelitis* appears under *Remarks*, the animals were etherized and an autopsy performed. The statement applies to examination of sections of spinal cord.

TABLE I—*Continued*

Case	Monkey	Virus No.	Virus-serum mixture 1:1	Date of inoculation	Outcome	Neutralization	Remarks
44773 (mother) 19 yrs.	51-1	515	1.2	4/10/30	No symptoms	+	
44773 (baby)	30-1	515	1.2	4/10/30	No symptoms	+	
44831 (mother) 24 yrs.	38-3	515	1.2	4/10/30	No symptoms	+	
44831 (baby)	33-2	515	1.2	4/10/30	No symptoms	+	
Convalescent serum No. 83	54-3	515	1.2	4/10/30	No symptoms	+	
Normal monkey serum	34-6	515	1.2	4/10/30	4/18 Paralyzed	—	Typical microscopic lesions of poliomyelitis
44844 (mother) 26 yrs.	54-4	515	1.2	4/22/30	No symptoms	+	
44844 (baby)	54-5	515	1.2	4/22/30	No symptoms	+	
44830 (mother) 35 yrs.	54-6	515	1.2	4/22/30	No symptoms	+	
44830 (baby)	54-7	515	1.2	4/22/30	No symptoms	+	
44845 (mother) 21 yrs.	54-8	515	1.2	4/22/30	No symptoms	+	
44845 (baby)	54-9	515	1.2	4/22/30	No symptoms	+	
Convalescent serum No. 83	55-0	515	1.2	4/22/30	No symptoms	+	

TABLE I—*Concluded*

Case	Monkey	Virus No.	Virus-serum mixture 1:1	Date of inoculation	Outcome	Neutralization	Remarks
Normal monkey serum	55-1	515	cc. 1.2	4/22/30	4/28 Paralyzed	—	Typical microscopic lesions of poliomyelitis
44860 (mother) 22 yrs.	47-0	515	1.2	5/ 8/30	No symptoms	+	Typical microscopic lesions of poliomyelitis
44860 (baby)	47-6	515	1.2	5/ 8/30	No symptoms	+	
44849 (mother) 23 yrs.	53-8	515	1.2	5/ 8/30	No symptoms	+	
44849 (baby)	49-8	515	1.2	5/ 8/30	No symptoms	+	
44856 (mother) 22 yrs.	49-6	515	1.2	5/ 8/30	5/25 Paralyzed	±	
44856 (baby)	53-6	515	1.2	5/ 8/30	No symptoms	+	
Convalescent serum No. 83	50-8	515	1.2	5/ 8/30	No symptoms	+	
Normal monkey serum	47-3	515	1.2	5/ 8/30	5/16 Paralyzed	—	Typical microscopic lesions of poliomyelitis

through transmission from the milk in suckling, nor has the duration of passive maternal immunization been determined. But the fact that eleven out of fifteen (73.5 per cent) children from 9 months to 5 years of age failed to neutralize the virus (2) (two of them were 9 months and 11 months old respectively) together with the fact that as in the case of diphtheria the incidence rises sharply after 6 months of age, suggests the probability that the mechanism of maternal immunization to poliomyelitis is similar to that of diphtheria.

At this point we may discuss briefly the results of these and previous tests for immunity to poliomyelitis from the point of view of their statistical significance. The tests performed on individuals of any one category is small—too small, as we have repeatedly pointed out, to establish the point in question when taken alone. But even where samples are small, if we observe several such samples and find them consistent with each other and with other comparable observations, the statistical significance of such observations rises rapidly.

In the first place, as discussed in detail in our earlier communications regarding the neutralization test, the test may be considered as a reliable indication of immunity.

The idea that the majority of urban adults have become immunized to the virus of poliomyelitis, upon which our present conception of the

TABLE II

Summary of Neutralization Tests for Virus of Poliomyelitis—Urban Adults

	Immune	Per cent	Not immune	Per cent
Northern adults.	7	85.7	1	14.3
Southern adults.	19	90.5	2	9.5
Northern mothers.	10	83.3	2	16.7
Total urban adults.	36	87.8	5	12.2

epidemiology of the disease hinges, is not only supported by tests which show immunity in increasing frequency up to adult age but by three separate sets of tests on urban adults themselves, all of which, as shown in Table II, are in close agreement.

The curves for immunity in normal individuals at different age groups both in urban and in rural populations have a similar trend. The diminished extent of immunity in a rural population is in accordance with diminishing chances of contact transmission of the virus, due to decrease in density of population.

The extent of immunity at different ages and in urban and rural populations, as well as in mothers and infants, is in accord with what might be expected from an analysis of the age distribution of the disease.

Finally, the extent of immunity to poliomyelitis in each age group

tested corresponds closely with that of immunity to diphtheria. This with other evidence that the process of immunization is essentially the same for the two diseases is regarded as additional support for the statistical validity of the relatively small number of observations which have been made on poliomyelitis.

SUMMARY

Neutralization tests for the virus of poliomyelitis on blood serum of urban mothers and their newborn infants showed that immunity was present in ten out of twelve (83 per cent) infants and in ten out of twelve (83 per cent) mothers, with a complete correspondence between mother and infant. These tests point to passive transmission of immunity from mother to infant. Previous tests on other children (1 to 5 years) indicate that immunity in infants is transitory. Previous observations concerning the extent of immunity in urban adults are confirmed and extended. The results of these tests are in accord with the age distribution of poliomyelitis and parallel corresponding observations in diphtheria.

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CULTIVATION OF VACCINE VIRUS

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Many investigators have cultivated vaccine virus in bits of living tissue embedded in plasma. These small cultures were made on cover-slips which were then inverted and sealed over hollow ground slides. For many reasons methods of making larger cultures are highly desirable. To this end, Carrel and Rivers (1) devised a procedure whereby cultures of vaccine virus were made in 2 to 8 cc. amounts. This was accomplished by inoculating minced chick embryo tissue with vaccine virus and then embedding it in diluted chicken plasma in Carrel flasks. This method was improved upon by Maitland and Maitland (2) who used a fluid medium consisting of minced chicken kidney suspended in chicken serum diluted with Tyrode's solution. Later Muckenfuss and Rivers (3) altered Maitland's medium by substituting rabbit testis and serum for chicken kidney and serum. The fact that certain viruses, *e.g.*, vaccine virus, Virus III, herpetic virus, will multiply in a medium of this nature has been amply verified by the work of Eagles and McClean (4), Andrewes (5-7), and Maitland and Laing (8).

The work to be described at this time has to do with the simplification of the technique for the cultivation of vaccine virus suitable for Jennerian prophylaxis in man. A medium, in which tissues and serum from halfgrown or adult animals are used, may easily permit the entry of an unknown virus into the cultures as a contaminant. The chance of such an occurrence can be lessened by the use of minced chick embryo suspended in Tyrode's solution. The possibility that such a medium would be appropriate was suggested by an experiment in which Andrewes (6) showed that Virus III survived and formed inclusions in rabbit testicular tissue suspended in Tyrode's solution.

Methods and Materials

Virus.—Levaditi neurovaccine virus that had been propagated for 6 months in rabbit testicles was used to initiate the cultures.

Tissues.—Testicular tissue was obtained aseptically from rabbits, washed in Tyrode's solution, placed in a sterile watchglass contained in a Petri dish, minced with scissors, and then distributed in proper amounts into flasks by means of a pipette. Embryo tissue was obtained from eggs, incubated 9 to 12 days, that had been opened according to the method of Carrel and Rivers (1) or that of Eagles and McClean (4).

Fluids.—When serum was used it was collected from rabbits, and diluted with 3 parts of Tyrode's solution. Tyrode's solution, pH about 7.2, prepared according to the following formula was sterilized by filtration: NaCl, 8 gm.; KCl, 0.2 gm.; CaCl₂, 0.2 gm.; MgCl₂, 0.1 gm.; NaHPO₄, 0.05 gm.; NaHCO₃, 1.0 gm.; glucose, 1.0 gm.; water *q.s.* 1000 cc.

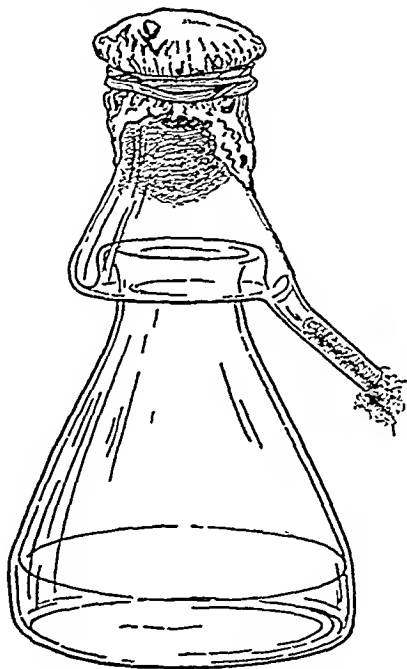
Containers.—Containers for the production of vaccine virus in large amounts must be of sufficient size and easy to handle. Furthermore, they must permit aeration and prevent contaminations and evaporation. At first, cultures were made in Carrel D flasks. Then 50 cc. Erlenmeyer flasks, stoppered with cotton over which two layers of tinfoil were placed, were used. Neither type of container was found entirely satisfactory for the work. Finally a flask was designed (collar flask) which proved to be suitable. The mouth of the flask (Text-fig. 1) is of sufficient size to permit the removal of the contents by means of a pipette. It is closed by a cotton plug over which are stretched two or three layers of tinfoil. The enlargement in the neck reduces the amount of water of condensation on or near the cotton plug and also prevents the water that does touch the cotton from running down and contaminating the culture. The small opening, plugged with cotton, on the side serves for ventilation. The bottom is perfectly flat, thus providing a large area over which a thin layer of culture may be evenly distributed. The flasks are made in two sizes. The dimensions of the large one are: height, 14 cm., diameter of mouth, 3.5 cm., diameter of base, 9 cm. The measurements of the small one are: height, 9 cm., diameter of mouth, 2.5 cm., diameter of base, 4.5 cm. In the large flasks, 2 to 4 gm. of tissue suspended in 15 cc. of Tyrode's solution were used, while in the small ones 0.5 to 1 gm. of tissue suspended in 5 cc. of Tyrode's solution was employed.

Preparation of Cultures.—Approximately 1 gm. of tissue was used to each 5 cc. of diluted serum or Tyrode's solution. Testicular or chick embryo tissue suspended in its vehicle was distributed in flasks. Prior to use, the sterility of the medium was tested on blood agar and in broth. During this interval (48 hours), the prepared flasks were stored at +5°C. The medium was then inoculated with 0.1 to 0.2 cc. of virus emulsion, and the cultures were incubated at 37°C. for 7 days. New cultures were made by the direct transfer of a few drops of the old culture into flasks of fresh medium. In this simple manner, vaccine virus can be propagated through an indefinite number of culture generations.

Titration of Virus.—Cultures for titration were ground in sterile mortars, and then appropriate dilutions were made with Locke's solution. A fresh pipette was always used for each successive dilution. 0.25 cc. of each dilution was injected into the shaved skin of rabbits. Daily observations of the animals were made for a week.

EXPERIMENTAL

In our investigation concerning the cultivation of vaccine virus, many experiments have been performed. All of them will not be



TEXT-FIG. 1. Type of flask used for the cultivation of vaccine virus

described, inasmuch as some are similar to experiments made and reported by others (8). Part of our work, however, is different from that previously described and the results are summarized in Chart 1.

The first cultures of Series A (Chart 1) were made February 17, 1930. A medium consisting of rabbit testicular tissue and a mixture of rabbit serum (1 part) and Tyrode's solution (3 parts) was inoculated with

vaccine virus and then distributed in Carrel D flasks. Virus from a culture of the third generation of Series A was inoculated into a flask (Series B) of medium comprised of chick embryo tissue suspended in

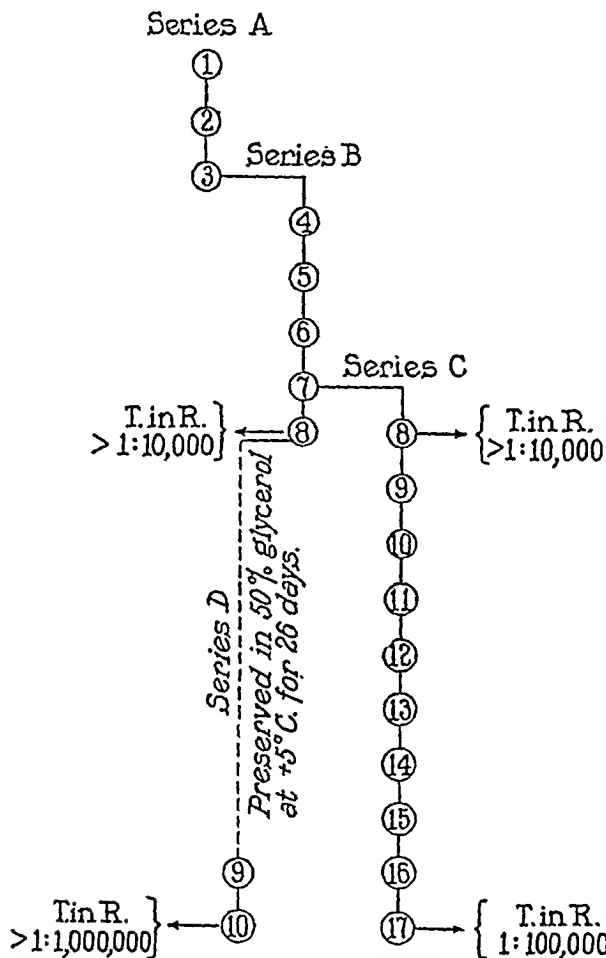


CHART 1. Summary of experiments on cultivation of vaccine virus.

The media used in the different series were as follows: Series A: Rabbit testicular tissue in a mixture of rabbit serum and Tyrode's solution. Series B: Chick embryo tissue in a mixture of rabbit serum and Tyrode's solution. Series C and D: Chick embryo tissue in Tyrode's solution.

T. in R. indicates titer in rabbit.

rabbit serum (1 part) and Tyrode's solution (3 parts). Seventh generation virus (Series B) was inoculated into a medium of chick embryo tissue suspended in Tyrode's solution (Series C). At this time collar flasks were first employed and since then they have been in general use.

The virus in Series C has been propagated for 10 generations, and is still under cultivation.

The fact that the virus multiplied is obvious and a mathematical proof seems superfluous. Nevertheless, one estimation will be given. The titer of the eighth generation virus (Series B) was $>1:10,000$. This virus mixed with glycerol was stored at $+5^{\circ}\text{C}$. for 26 days. Then the preserved virus was diluted 1:100 with Tyrode's solution, and 5 drops of the dilution were inoculated into 5 cc. of fresh medium. After a 9-day period of incubation, 4 drops of this culture were added to 15 cc. of fresh medium. After incubation the titer of the last culture was 1:1,000,000 (Series D).

From the work summarized in Chart 1, it is obvious that the virus can be transferred without difficulty from one kind of medium to another. Furthermore, culture virus, mixed with glycerol, sealed, and stored at $+5^{\circ}\text{C}$., can be preserved for a long time. In this manner virus has been preserved for 64 days without great loss in potency. Moreover, the preserved virus can be employed as an inoculum for the initiation of fresh cultures.

The virus propagated in cultures for a long time seems to have lost none of its characteristics, for, when it is spread on the scarified shaved skin of a rabbit, typical vaccinal lesions occur. Moreover, serum from a rabbit immune to Levaditi's neurovaccine virus completely neutralizes the culture virus. Finally, an immune serum prepared with the culture virus neutralizes the Levaditi vaccine virus.

DISCUSSION

A simple method for the *in vitro* cultivation of vaccine virus has been devised. The flasks employed are easy to manipulate and permit the production of large amounts of virus. Moreover, the medium is innocuous and inexpensive, consisting of minced chick embryo suspended in Tyrode's solution. In spite of the simplicity of the procedure, however, one should not forget the fact that living susceptible host cells are employed. No one has as yet been able to demonstrate that vaccine virus can multiply in the absence of such cells.

The facility with which vaccine virus can be handled and cultivated *in vitro* varies with the strain. This fact has been evidenced by previous experiences in our laboratory and also in that of Eagles and

McClean (4). In the work reported at this time a neurovaccine virus was employed. Studies have been planned to determine whether it is possible to adapt a dermovirus to the type of medium used by us.

SUMMARY

1. A strain of neurovaccine virus was cultivated in a medium consisting of minced chick embryo suspended in Tyrode's solution.

2. The virus upon cultivation apparently lost none of its essential characteristics.

3. The culture virus can be preserved and stored for long periods of time. Furthermore, the preserved virus can be used to initiate fresh cultures.

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THE STANDARDIZATION OF ANTIPNEUMOCOCCIC SERUM TYPES I AND II

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The mouse protection test for establishing the value of antipneumococcic serum does not give consistent results unless a large number of mice are used. Friedlander, Sobotka and Banzhaf (1) used the specific polysaccharide of the pneumococcus as an antigen for precipitation tests, to determine the potency of serum. Their findings agreed fairly well with the mouse protection test of Felton (2).

In confirming the work of Heidelberger and Avery (3), on the pneumococcus polysaccharide, we were struck by the consistent results obtained in successive precipitation tests with any one serum. At the same time, we have long been aware of the difficulty in the interpretation of the mouse protection test in judging the potency of antipneumococcic serum. We, therefore, have undertaken parallel studies on a series of antipneumococcic serums, using both the specific polysaccharide precipitation and mouse protection tests. A close agreement between the results of the two methods was found to exist.

The difficulty in the preparation of polysaccharide of the high purity obtained by Avery and Heidelberger would be a barrier to the practical routine application of the polysaccharide precipitation test. We found, however, in comparing different lots of polysaccharides of varying purity, that results were comparable with each lot.

Crude polysaccharide prepared by the following technique was found very satisfactory for use as antigen in the precipitation test:

An 8 day broth culture of the type pneumococcus is evaporated to about one-fifteenth the original volume. The material is chilled and trichloroacetic acid crystals added to make a 5 per cent solution. A precipitate is formed and removed by centrifugation as soon as possible. To the clear supernatant about one and one-half volumes of alcohol are added and centrifuged. The alcoholic pre-

precipitate contains the polysaccharide which is soluble in water and is purified by repeated re-precipitations with alcohol. The final precipitation is usually done with acetone. Further purification does not seem necessary.

Polysaccharide made by this method when dried and dissolved in saline 1 to 10,000 can be satisfactorily used for antigen in the precipitation test.

The technique of the precipitation test used by us is as follows: Equal volumes of serum dilutions and a constant polysaccharide dilution (1 to 10,000) are mixed and placed in a water bath at 37°C. The water level should be not more than one-half that of the test mixture. The test is read after 2 hours. Further precipitation in higher dilutions may occur after longer incubation at 37°C., or a subsequent period on ice, but the result is not as clear cut, variations being more frequent than when the 2 hour reading is used.

The interpretation of the test is based on a comparison of the titer of the complete precipitation of a standard serum, F-146 (supplied by Felton), as containing 200 protective units* per cubic centimeter for *Pneumococcus* Types I and II, with that of the unknown test serum.

In repeated tests, using a solution of Type I polysaccharide of 1 to 10,000, the standard serum gave complete precipitation in a dilution of 1 to 20. On this basis the number of units for Type I of the test serum would be expressed by the following equation.

$$\frac{\text{Dilution of test serum}}{20} \times 200 = \text{Number of units of the test serum.}$$

The results of comparative mouse protection and polysaccharide precipitation tests with a portion of the many polyvalent antipneumococcic Type I and II serums, tested, are shown in Table I. Only results of tests with Type I are given. Type II values can also be tested by the polysaccharide precipitation test, the equation of calculation, however, being different as the standard serum F-146 with a protective unit value of 200 units in Type II, results in a complete precipitation at a dilution of 1:35 with the specific Type II polysaccharide used by us.

* The standard value of this serum has recently been changed by Felton after testing with the different strains of pneumococcus used by the various laboratories, to 500 units per cubic centimeter Type I and Type II.

The interpretation of the mouse protection by direct calculation for unit value according to virulence is frequently difficult. The variations and irregularities in the time of the deaths frequently make necessary a repetition of the test before results are obtained which satisfactorily check with the established value of the standard serum.

TABLE I

Type I Antipneumococcus Protective Units Estimated by the Mouse Protection and Polysaccharide Precipitation Tests

Serum	Mouse test	SSS Ppt.
F-146	200	200
7892-N	100	120
7879-A	80	80
7895-G	200	220
7899-M	100	100
7900-H	50	80
7905-I	300	300
7908-I	300	300
8031-A	40	50
8032-A	50	50
MM8843-A	600	560
MM8843-B	100	90
MM8843-C	50	20
46130	50	50
48398	400	350

* "SSS Ppt." is the designation used by Heidelberger and Avery for the "soluble specific substance"—polysaccharide responsible for the precipitate.

The unit values given in Table I are therefore calculated by direct comparison with the 200 unit standard serum F-146.

Table II shows the results obtained with the polysaccharide precipitation and mouse protection test, on using serum from consecutive bleedings from horses under immunization. The protection test used in this series was that endorsed by the Hygienic Laboratory and controlled by their standard serum. The serums are classified as *good*, *fair* or *poor*, according to the degree of protection, expressed in percentage, obtained in the group of mice placed on test. The ease with which one can be misled by the use of the mouse test alone is shown. For example in Serum 7892 the value of the bleeding of 2-15-30 and of

3-15-30 is more accurately determined by the polysaccharide precipitation test. The results of tests on bleedings which prove horses to be producing serum of a higher value—such as Serum 7905—are consistent with the mouse protection test. The exact status of serum from horses on the borderline of productivity is much more accurately

TABLE II
Antipneumococcic Type I
Protective Units in Consecutive Horse Bleedings

Date	SSS Ppt.	Mouse	Date	SSS Ppt.	Mouse
<i>Serum 7879</i>			<i>Serum 8083</i>		
1-28-30	90	Fair	12-26-29	180	Fair
2- 1-30	80	Good	1-26-30	100	Fair
2-15-30	50	Fair	2-10-30	150	Fair
3- 7-30	30	Poor	3- 7-30	80	Fair
3-31-30	30	Fair	3-15-30	100	Fair
			4- 1-30	80	Fair
<i>Serum 7892</i>			<i>Serum 8110</i>		
1-30-30	180	Good	12- 4-30	—	Poor
2-15-30	220	Fair	1-30-30	240	Good
3- 1-30	100	Fair	2-15-30	180	Good
3-15-30	90	Fair	3- 7-30	30	Fair
			3-22-30	50	Fair
<i>Serum 7905</i>			<i>Serum 8113</i>		
1-15-30	200	Good	9-25-29	—	Poor
1-30-30	300	Good	12- 4-29	—	Fair
2-15-30	280	Good	1-30-30	180	Good
3- 1-30	280	Good	2-15-30	250	Fair
			3- 1-30	280	Good

determined by the polysaccharide precipitin test, as shown in bleedings of Serums 8083, 8110 and 8113.

At present the immunization of all horses used in the production of antipneumococcic serum is followed by means of the precipitation test. Mixtures of bleedings for concentration or other purposes, are checked with the mouse protection test.

DISCUSSION

The standardization of antipneumococcus serum Types I and II has been a very difficult problem on account of the variable results obtained with the mouse protection test. The difference in the virulence of the cultures used, of *Pneumococcus* Types I and II makes comparison of the mouse tests of various laboratories impossible. Even within one laboratory, in which the same strain is always used, and with every care for absolute duplication of method and accuracy of technique, variations occur which often make the interpretation of the test difficult, and costly repetition necessary. This is due to the very nature of the test, susceptibility of the mouse to a virulent pneumococcus being so marked that the smallest deviation in the number of organisms injected into the test animal leads to most confusing irregularity in the time of death. Also variation in number of organisms is unavoidable by the method because of the procedure of making dilutions as a basis for dosage.

We have found that the precipitation test is practical and gives comparable results on the repetition of tests. Similar conclusions have been reached by Heidelberger, Sia, and Kendall (see the following paper (4)).

It seems highly probable that in the case of organisms elaborating specific polysaccharides this method of testing can be used for the standardization of the antiserums. We have succeeded with anti-anthrax serum, the standardization of which has been very unsatisfactory, and we are proceeding with tests of other antibacterial sera.

CONCLUSIONS

1. The mouse protection test used for the standardization of antipneumococcic serum Type I and II is very unreliable.
2. The specific polysaccharide precipitation test can be used to establish the comparative value of a test serum to a standardized serum.
3. The polysaccharide used for such tests does not have to be of the highest purity.

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SPECIFIC PRECIPITATION AND MOUSE PROTECTION IN TYPE I ANTIPNEUMOCOCCUS SERA*

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Specific precipitation has long been known to occur in antipneumococcus sera showing marked protective power in mice (1), but it has been possible only recently to obtain indications of a parallel variation in these two properties. Thus Friedlander, Sobotka and Banzhaf (2) showed that the number of mouse protection units in certain Type I and Type II antipneumococcus sera varied in the same sense as the "precipitin index." In the preceding communication Zozaya, Boyer, and Clark (3) have shown that it is possible by means of the precipitin test to obtain within 2 hours numbers which agree excellently with the mouse protection units found in fifteen sera.

The great advantages of a rapid *in vitro* test for the potency of antipneumococcus sera over the tedious, expensive, and often uncertain mouse protection test had led the writers to search for a possible relation between specific precipitation and mouse protection in Type I antipneumococcus sera on the basis of their recent quantitative study of the reaction between the specific polysaccharide of Type III pneumococcus and its homologous purified antibody (4) as well as on the basis of almost completed data on the corresponding Type I reaction. While this work has not resulted in so simple and rapid a routine test as that of Zozaya, Boyer, and Clark, the writers feel that it establishes on an experimentally verified and theoretically reasonable basis a definite parallel between maximum specific precipitation in Type I

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antipneumococcus sera and mouse protection. Furthermore, this parallel can be drawn with sufficient exactness to warrant the statement that in the group of widely varying sera studied under the definite empirical conditions described below, sera containing 7 mg. or more of specifically precipitable protein per cubic centimeter showed a mouse protection value of 1000 units and over. It is suggested that should these, or similar values be found by other workers to hold for sera at their disposal, a quantitative grading of Type I antipneumococcus sera on the basis of their content of specifically precipitable protein would not only be feasible, but would also be far quicker, cheaper, and more susceptible of duplication in other laboratories than the present generally accepted mouse protection values.

EXPERIMENTAL

1. *Type I Pneumococcus Specific Polysaccharide.*—The Type I "soluble specific substance" used was prepared as in a preceding paper (5). Since an ash-free preparation of highest purity is not essential, an abbreviated method is here given.

20 liters of 5-day Type I pneumococcus culture in meat infusion phosphate broth containing 0.3 per cent of glucose are autoclaved and concentrated on the water bath to a volume of 1 liter. The concentrate is precipitated with 1.4 liters of alcohol and put through the initial 3-layer separation (6). The middle layer is treated with 200 to 250 cc. of hot water until all lumps are dissolved, and the mixture is centrifuged and the precipitate washed twice with small amounts of water. The clear solution and washings are chilled and acidified strongly to Congo red with 1:1 hydrochloric acid, centrifuging off in the cold and washing with a little 0.01 normal hydrochloric acid any precipitate formed at this point. The solution, the volume of which should not exceed 500 cc., is stirred vigorously, precipitated with 1.5 liters of chilled alcohol, and allowed to stand in the cold over night. The precipitate, after centrifuging, is taken up in water, with the addition of enough sodium hydroxide to maintain alkalinity. Water is added until all of the specific substance is in solution, and the remaining trace of insoluble material is centrifuged off. The supernatant, at a volume of 100 to 125 cc., is treated with 10 gm. of sodium acetate, and when this has dissolved, is chilled, precipitated with 70 cc. of cold alcohol, allowed to stand in the cold over night, and centrifuged in the cold. The precipitate is redissolved in 200 cc. of water, the solution is centrifuged, and the supernatant acidified with glacial acetic acid to pH 3.4, at which point the specific polysaccharide flocculates readily and is centrifuged off.

The supernatant should contain very little specific substance, but if it does, the pH should be adjusted in the direction of further flocculation and the precipitates combined. The specific substance is taken up in 100 cc. of chilled 0.05 normal acetic acid, centrifuged off again, and washed in the centrifuge bottle, first with redistilled alcohol and finally with redistilled acetone. It is then filtered on a Buchner funnel, washed with redistilled acetone, and dried *in vacuo* at room temperature. The yield in a typical instance was 0.8 gm.,* containing 0.5 per cent ash. Other analytical data, calculated to the ash-free basis, were: $[\alpha]_D^{299}$, N, 5.0 per cent, values practically identical with those of the best preparations previously reported (5). If larger amounts of broth are worked up it is best to repeat each precipitation.

2. Titration of Sera by the Precipitin Method.—If, on addition of a 1:10,000 saline solution of the specific polysaccharide to a portion of the serum, precipitation is rapid and heavy, 0.5 cc. of the serum should be used for the quantitative determination. This will suffice for all except very low grade sera, of which it is better to use 1 cc. The sera should be measured in duplicate with accurately calibrated pipettes into wide agglutination tubes (dimensions of 10 x 75 mm. have been found suitable). If 0.5 cc. serum has been used, 1 cc. saline is then added to each tube, followed by 0.5 cc. of a saline solution containing 1 mg. of the specific polysaccharide per cubic centimeter, to make a total volume of 2 cc. Ordinary uncalibrated pipettes are adequate for the saline and polysaccharide. The tubes are plugged and the contents carefully and thoroughly mixed by a rotary motion imparted by drawing the finger tips rapidly and repeatedly diagonally down the side of the tube. After the tubes have been allowed to stand 2 hours in the water bath at 37° and over night in the icebox,† the plugs are removed and the tubes centrifuged for 10 minutes at 1000 revolutions per minute, either in a refrigerating centrifuge‡ or immersed in ice-water. The supernatant liquid is then carefully drained off by inverting the tube and wiping the mouth of the tube with filter paper after a few minutes. The tubes are then placed in ice-water and the precipitates are each washed with 2 cc. of an ice-cold 1:20,000 saline solution of the specific polysaccharide, mixing the contents as before. It appears to make little difference whether or not the disc of precipitate is loosened from the bottom of the tube. After $\frac{1}{2}$ hour in the cold the tubes are again centrifuged and drained

* Since the isoelectric substance is insoluble, solutions must be made up with the aid of alkali.

† Sterile technique should be employed up to this point. In the case of low grade sera identical values are obtained if the tubes are allowed to stand in the water bath only $\frac{1}{2}$ hour, followed by $\frac{1}{2}$ hour in ice-water before proceeding. In more potent sera results a few tenths of a milligram per cubic centimeter too low are obtained by shortening the process in this way.

‡ Supplied by the International Equipment Co., Boston, Massachusetts.

as before. About 0.5 cc. of water is then added to each tube, shaking as before until the precipitate is loosened, after which the disc is dissolved by the addition of 2 drops of normal sodium hydroxide solution with rotation of the tube until the precipitate has disappeared. If the disc is allowed to stick to the glass, solution is much slower. The solution is then rinsed quantitatively into a micro-Kjeldahl flask or tube and the nitrogen determined by any standard procedure. The Pregl method, slightly modified, was used in the present work. Nitrogen found $\times 6.25$ = specifically precipitated protein.

3. *Mouse Protection Tests.*—For the biological estimation of the amount of protective antibodies contained in various samples of Type I antipneumococcus sera, the method described by Felton (7) was employed. According to this method, a *unit* (of antipneumococcus protective antibody) is defined as "that fraction of a cc. of serum which will protect against one million fatal doses of an 18-hour serum broth culture. The culture used must be of such virulence that 3 to 10 organisms when injected intraperitoneally into a mouse kill the mouse in from 36 to 48 hours."

Briefly, the method consists of the intraperitoneal injection into white mice of 0.5 cc. of a 1:200 dilution* of an 18-hour serum broth culture of the Type I pneumococcus mixed with an equal amount of the dilution of the Type I antipneumococcus serum to be tested. The immune serum dilutions were made in sterile normal saline and the dilutions used were 1:25, 1:50, 1:100, 1:200, 1:400, 1:600, 1:800, 1:1000, 1:1200, 1:1600, no attempts being made to titrate more closely by the use of intermediate dilutions. The organism and the immune serum dilutions were taken up into a syringe and injected as soon as possible intraperitoneally into mice. For each serum dilution tested, mice were injected in triplicate, and observed for 96 hours. The highest immune serum dilution protecting two out of the three mice for a period of 96 hours was taken as the protective unitage of the serum.

The results obtained are given in Table I.

DISCUSSION

In the course of a quantitative study of the precipitin reaction between Type I pneumococcus specific polysaccharide and purified Type I pneumococcus antibody, it was found that maximum precipitation occurred when the initial concentration of specific polysac-

* Calculated to contain 500,000 lethal doses.

Specific Precipitation and Mouse Protection in Type I Antipneumococcus Sera

Sample No.	Source	Agglutination titer	Units found in individual mouse protection tests	Individual determinations (in duplicate) of sp. pptble. protein	Average mouse protection units	Average specifically pptble. protein
B108C	4*	1:10	50	<i>ms. per cc.</i> 0.7 0.6	50	<i>ms. per cc.</i> 0.7
101	1 (diagnostic)	1:60	100 100 100	1.7 1.7 1.7	100	1.7
110	5 (standard)**		200	1.9	200	1.9
108B	4*	1:40	200	2.0 2.0	200	2.0
105	3 (diagnostic)	1:60	800 400 800 400 600†	3.1 3.3 3.4	600	3.3
108A	4*	1:40	600	3.8 3.8	600	3.8
107	3 (diagnostic)		800	5.0 4.8	800	4.9
103	3 (therapeutic)	1:120	1200 1000 1000 800	6.9 6.5 6.7 6.6	1000	6.7
104	2 (diagnostic)	1:160	>1200‡ 1200 >1600‡ 800	7.0 6.9 7.4 7.1 6.9	1200	7.1
102	1 (diagnostic)	>1:120	>600‡ >800‡ 1200	7.4 7.9 7.6	1200	7.6
106	3 (therapeutic)	1:160	>1200‡ >1600‡	9.7	1600	9.7

* Number 8843 in the paper by Zozaya, Boyer and Clark.

** Number F 146 in the paper by Zozaya, Boyer and Clark.

† This dilution was not used until the first four results had been obtained.

‡ Highest dilution tested.

charide was 1:2,000. Since, however, all but a small fraction of the specifically precipitated protein is carried down at one-half this concentration, the latter strength is used in the test herein given, in the interest of economy of specific substance. Similarly, the precipitate is washed with a 1:20,000 solution of the polysaccharide as this concentration is adequate to prevent appreciable dissociation of the precipitate and the nitrogen content of a solution of this concentration is too small to interfere.

It can not be claimed that the nitrogen figures obtained are more than an approximate representation of the specifically precipitable protein in the sera tested, since a single washing of the compact disc-like precipitate would scarcely remove all entrained inert serum proteins. On the other hand, washing with 1:20,000 polysaccharide solution, as is done in the test, probably fails to prevent absolutely dissociation of the precipitate formed under optimum conditions, so that these two errors tend to balance each other. Since duplicates and repeated determinations usually check, at least consistent results are readily obtained. Moreover, any number of sera may be tested at a time.

From Table I it will be seen that the sera tested, when arranged in the order of increasing specifically precipitable protein per cubic centimeter, are also found to be in the order of increasing mouse protection units. By the methods employed, 7 mg. of specifically precipitable protein per cubic centimeter corresponded roughly with a mouse protection value of 1000 units, and it is felt that an easily reproducible and readily determinable standard system of grading could be worked out along these lines.

SUMMARY

1. A rapid and simple method is given for the approximate determination of the specifically precipitable protein in Type I antipneumococcus sera.

2. It is shown that a close parallel exists between the specifically precipitable protein and the number of mouse protection units in a wide variety of Type I antipneumococcus sera.

3. Owing to the consistent results obtained and the rapidity, simplicity, and economy of the method, its use is proposed instead of the

mouse protection test as a basis for the titration of standard sera and the comparison of others with a standard.

4. A method is given for conveniently preparing highly purified specific polysaccharide of Type I pneumococcus.

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THE EFFECT OF INFLAMMATORY REACTIONS ON TISSUE IMMUNITY

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PLATES 18 AND 19

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The problems of immunity in the past have been approached chiefly by noting alterations in the blood plasma. Although it has long been recognized that the invaded tissues also play a significant rôle in resistance, the difficulties encountered in studying this mechanism have greatly retarded a complete understanding of the problem. Most of the studies on cellular immunity have been devoted to various aspects of tuberculosis, for in this disease true humoral immunity is strikingly absent. Only recently has it been adequately appreciated that most "normal individuals" harbor a variety of pathogenic organisms in chronic foci in the naso-pharynx or elsewhere in the body and derive from them a generalized alteration of tissue response quite analogous to that found in tuberculosis (1). This bacterial allergy is usually demonstrated by injecting small amounts of broth filtrates of the organism intradermally and noting the degree of redness and infiltration that has developed 24 hours later. The skin reactions are not type specific and may be demonstrated for many organisms biologically unrelated. The relationship of tissue reactivity to bacterial invasion and the development of disease is obviously difficult to study in the human being. Consequently we have transferred our investigations to rabbits using as our infecting agent *B. lepticum* which is harbored in the naso-pharynx and is the cause of the majority of acute and chronic lesions of the respiratory tract in this convenient laboratory animal (2, 3, and 4). Infections of the skin chiefly have been employed because here it is easiest to observe the progress of the lesion.

Before undertaking any experiments it has been our routine procedure to skin-test the animals with 0.2 cc. of a 48-hour broth filtrate of *B. lepi-septicum*. Readings were made 24 hours later and the animals classified, according to the size, redness and induration of the injected skin, into the following groups: (1) "negative reactors," (2) "weak reactors," (3) "moderate reactors" and (4) "strong reactors." This classification has proved important because the strong reactors show a definite tendency to localize the infection, even when the serum in such animals (in those animals tested) shows no agglutinins or demonstrable protective substances against the organism used.

TABLE I

Comparative Infections in Strong Reactors and Weak Reactors

	Total infection mortality	Died		Survived	
		Large lesion	Small lesion	Large lesion	Small lesion
Strong reactors.....	9	7	2	5	13
Total number, 27.....	(33.3%)	(25.8%)	(7.4%)	(18.5%)	(48.1%)
Weak reactors.....	40	34	6	3	10
Total number, 53.....	(75.5%)	(64.1%)	(11.3%)	(5.6%)	(18.8%)

Contrasted Infections in Weak and Strong Reactors

The negative and weak reactors, unless serological immunity is present, show but slight ability to limit the spread of the local lesion. After the injection of about 0.1 cc. of a light suspension of a virulent strain of *B. lepi-septicum* (R. D.) into the skin of the flank, the characteristic infection begins as a small, pale papule at the site of inoculation and is well developed in 24 hours. Hemorrhagic necrosis appears quickly and from its intensity may be predicted the severity of the infection which will subsequently develop. Within from 5 to 10 days the lesion spreads downward over the abdomen forming a large, black oedematous mass. The animal becomes feverish and frequently dies. Post mortem examination usually reveals a positive blood culture but seldom metastatic lesions.

The strong reactors may occasionally develop similar large lesions

and succumb to the infection; in such cases they quickly lose all skin reactivity, hemorrhagic necrosis appears and the progress of the disease is in every way identical to that in the poor reactors. More frequently, however, the site of the infection is larger and redder after the first 24 hours and spreads but little thereafter. Histologically, the typical lesions of the strong reactors show more leucocytes, both of the polymorphonuclear and monocytic variety, and much less cellular disintegration than those of the weak reactors.

In Table I is shown the outcome of comparable infections in a series of eighty rabbits. It is obvious that there is a higher degree of resistance among the strongly reacting groups, this immunity being manifested chiefly by lesions more limited in extent. It might be argued that the presence of allergy presupposes a past "infection experience" from which a generalized immunity might also be derived. Evidence for this cannot be demonstrated in the serum of these animals. It therefore appears that the immunity furnished is *cellular* in nature and the present work is reported in an attempt to analyze some of the factors in such a type of immunity.

Infection of Skin Areas Previously Infiltrated with Bacterial Filtrates

Much study has been devoted to the fate of various pathogenic bacteria when injected into inflamed tissues. The results reported depend upon the organisms used, the irritant employed, and the duration and intensity of the inflammation before the infecting agent is introduced. An excellent summary of the important work on this subject has recently been made by Opie (5).

Besredka (6) observed that filtrates of certain bacteria applied to the skin rendered the skin immune to these bacteria and suggested the use of these filtrates therapeutically. Gay (7) and his co-workers have shown that a non-specific sterile inflammation which caused an increase of clasmotocytes protected the pleura of rabbits against streptococcus. Rivers and Tillett (8) and Mallory and Marble (9) have demonstrated a local protection of the skin to streptococcus and staphylococcus by infiltrating the dermis 24 hours previously with bacterial filtrates, or even plain broth, which suggests very strongly that there is no specificity to the phenomenon.

We have been able to demonstrate a local protection of the skin to virulent *B. lepietolicum* by infiltrating 24 hours previously with

filtrates of various strains of this organism. Equal protection, however, was obtained with filtrates of *B. coli*, *Streptococcus hemolyticus* and to a less extent with plain broth. When filtrates and organisms are injected into the tissues simultaneously, there is no demonstrable protection.

Normal rabbits showing weak skin reactions, and presumably susceptible to infection, were shaved over the flanks at least 48 hours before the experiments. An area of skin about 4 cm. in diameter was infiltrated with one of the above filtrates by injecting small amounts at adjacent points. The total quantity employed was usually 0.7 cc. 24 hours later slight redness and oedema were present depending upon the reactivity of the skin of the individual animal. Skin thus prepared was injected with 0.1 cc. of standard virulent strain of *B. leprosepticum* (R. D.). In some animals a similar injection was made in the opposite flank.

TABLE II

Effect of Infiltrating Skin with Bacterial Filtrates 24 Hours before Inoculation with Virulent B. leprosepticum

Total number of rabbits, 26	No necrosis		
	Infection localized to filtrate treated skin	9	34.7%
	No necrosis in filtrate treated area		
	Infection spread beyond filtrate area with usual necrosis	12	45.7%
	Necrosis present in filtrate treated skin	5	19.2%

In others no control injection was made in order to avoid the possible inhibiting effect of another lesion. Many of this group were infected intracutaneously several days later to demonstrate absence of generalized immunity.

The results summarized in Table II indicate roughly three different types of response:

1. A complete localization of the lesion to the infiltrated skin.
2. Spreading of the lesion with development of the usual large necrotic area over the abdomen, *but in which the skin showed no necrosis in the filtrate-treated portion.*
3. Lesions similar to the above (2) except for necrosis in the filtrate-treated portion. This necrosis was usually very slight except in one case. This animal received only broth infiltration before being injected.

Infection in the prepared skin often produces, within a few hours, a diffuse, red lesion in which the absence of necrosis is very striking and in which healing takes place more promptly than in control infections. This absence of necrosis was also noted by Mallory and Marble and appears to the writer to be of great significance, for we can assume that the inflammation surrounding a lesion is due to the diffusion of bacterial products resembling those found in filtrates. A non-specific mechanism for localizing certain infections might be assumed to exist if this zone of preparation antedates the bacterial invasion by sufficient time or if the animal possesses cells capable of rapid stimulation by these substances, as is the case in the allergic group.

Infection of Skin Areas into Which B. leprosepticum Immune Serum Has Been Infiltrated

The type of resistance just described must not be confused with that conferred upon an area of skin by injecting previously or simultaneously with the organisms a small amount of immune serum. We have employed for our experiments a stock mixture of sera obtained from rabbits convalescing from a large cutaneous infection caused by *B. leprosepticum*. This serum contains practically no agglutinins for the organism, but protects in minute amounts an area of skin infiltrated with it several hours before infection. In such cases the site of inoculation is hardly visible. When organisms and immune serum are injected simultaneously there is often a redness and induration at the site of injection which tends to heal rapidly and never develops into a formidable lesion, even in the most susceptible animal. In the last analysis the efficacy of immune serum in *B. leprosepticum* infection is its ability to protect the tissue cells of all varieties from bacteria and their injurious products.

Infection of Skin Areas Reacting to Various Chemical Irritants

Before analyzing the various factors involved in the localization of infection in animals showing no serum immune substances, it is important to contrast the effect of filtrates described above with that of various chemical irritants such as dilute acetic acid, croton oil and xylol.

Normal weak reactors were injected intradermally with small amounts of one of these substances. Within 24 hours a large red lesion developed with varying degrees of necrosis at the point of injection and often with puffy oedema over the dependent portions. Bacteria injected into such an area spread rapidly, produc-

TABLE III

Infection of Areas of Skin Inflamed by Chemical Irritants 24 Hours Previously

Animal number	Chemical irritant used	Amount of irritant	Severity of local inflammation	Result of infection of local inflamed area after 24 hours	Result of infection of a control area of normal skin after 24 hours
		cc.			
3-42	Acetic acid 2%	0.5	Severe	Very large, necrotic lesion	Not done
3-43	Xylol	0.5	Severe localized	Large, necrotic, spreading	Not done
3-53	Croton oil	0.1	Severe oedema	Very large, necrotic, spreading	Not done
4-72	Acetic acid 5%	0.1	Very slight	Moderate 3.5 x 3 cm., spreading	1 x 1 cm.
4-73	Acetic acid 0.25%	0.1	Very slight	Moderate 3 x 2.5 cm., spreading	1 x 1.5 cm.

Infection of Similar Areas in Immune Animals

2-45	Acetic acid 2%	0.5	Moderate oedema	Large 5.5 x 5 cm. No spread	Moderate 4 x 4 cm.
2-68	Acetic acid 2%	0.5	Very slight	Small 2 x 2 cm. No spread	Small 1.5 x 1.5 cm.
2-78	Croton oil	0.1	Severe oedema, moderate necrosis	Large 7 x 3 cm. No spread	Small 1 x 1 cm.
2-81	Croton oil	0.1	Moderate oedema, slight necrosis	Large 8 x 3 cm. No spread	Small 1.5 x 1.5 cm.
2-99	Xylol	0.2	Moderate lesion red, localized	Moderate 4 x 4 cm. No spread	2 x 2 cm.
3-02	Xylol	0.2	Moderate lesion red, localized	4.5 x 4 cm. No spread	Small 1.5 x 1 cm.

ing a large necrotic lesion which usually proved fatal within a few days. Even when the amount of irritant was very small the dissemination of bacteria seemed to be increased. 24 hours later the infected irritated site was redder and larger than a control infection made elsewhere in normal skin. However, like a filtrate-treated area, no necrosis appeared at the areas receiving small amounts and healing

began much sooner. This confirms the work of Gay and others that the intensity of the previous injury often determines the outcome of local infection. (See Table III.)

Animals immunized to *B. lepi-septicum* were treated similarly. Twenty-four hours after injecting an area with one of the above-mentioned irritants, the site was inoculated with *B. lepi-septicum*. The infection spread throughout the inflamed area producing a lesion proportional in severity to that of the preliminary reaction. The immune animals never became ill, the lesions did not spread beyond the limits of the chemical irritation, necrosis was not so extensive, the discharge from the lesion was more purulent and, most striking of all, healing began early and developed more rapidly at the site previously inflamed than in a control infection in the same animal.

Immune serum injected mixed with the irritant is quite effective in controlling an infection induced the next day in the irritated area. A small series of normal rabbits received 0.4 cc. immune serum mixed with 0.1 cc. croton oil or 0.5 cc. of 2 per cent acetic acid, and 24 hours later, the lesions were inoculated with organisms but showed only slight infiltration which healed rapidly in the portion receiving the immune serum, but outside this protected area showed the characteristic necrotic spread. This observation is of some interest when contrasted with the fate of immune serum injected with a protein antigen to be described in the next experiments.

Infection of Skin Areas Reacting to a Coagulable Protein to Which the Animal Has Been Previously Sensitized

When organisms are introduced into a wheal of reacting skin of an animal sensitized to a coagulable protein, a decreased tissue resistance is observed similar to that obtained when tissues are inflamed by chemical irritants.

Normal animals were sensitized to egg albumin by injecting 1 cc. of egg solution subcutaneously at 5-day intervals for 15 days. When 0.2 cc. of egg solution was then injected intradermally the familiar pink, oedematous lesion of hypersensitivity developed in 24 hours. Organisms injected into it spread with amazing rapidity throughout the whole reacting area, so that cultures made 18 hours later were strongly positive 10 cm. or further from the point of inoculation. Rapid necrosis appeared and the animals practically always succumbed. (See Fig. 1.)

The extent of the lesion in the first 24 hours was in proportion to the degree of hypersensitiveness shown by the animal.

We have studied the effect of immune serum on this type of infection and have obtained results which may throw some light on the nature of the underlying processes:

I. Infection of Egg-Reacting Sites in Animals Immune to the Infecting Organism

Rabbits were selected which had survived an infection with *B. lepi-septicum* and which had protective substances demonstrable in the serum in such concentra-

TABLE IV

Results of Infecting Areas of Egg Reacting Skin in Animals Immune to B. lepi-septicum and Sensitized to Egg Protein

Rabbit number	Intensity of skin reactivity to egg protein	Extent of infection in egg reacting site after 24 hours		Extent of infection in control site after 24 hours	
3-98	Marked oedema Sl. hemorrhagic	Large	8 x 4 cm.	Small	0.7 x 0.7 cm.
4-00	Marked oedema Mod. hemorrhage	Large	7 x 4 cm.	Small	1 x 0.7 cm.
2-72	Marked oedema	Moderate	6.5 x 5 cm.	Small	2 x 2 cm.
2-68	Moderate oedema Sl. hemorrhagic	Moderate	5 x 4.5 cm.	Small	1 x 1 cm.
2-66	Moderate oedema	Moderate	4.5 x 3.5 cm.	Small	3 x 2 cm.
3-87	Slight	Small	2.5 x 2 cm.	Small	1.5 x 1.5 cm.
3-88	Very slight	Small	1.3 x 1.2 cm.	Moderate	2.3 x 3 cm.
3-89	Very slight	Small	1.2 x 1.5 cm.	Small	1.5 x 1.3 cm.
3-99	Very slight	Small	1.7 x 1.7 cm.	Small	2 x 2 cm.

tion that 0.2 cc. injected into the skin of susceptible rabbits protected locally against a large number of *B. lepi-septicum* injected into the same site.

These immune animals were sensitized to egg albumin as outlined above, skin-tested with egg, and the reacting skin inoculated with virulent *B. lepi-septicum* 24 hours later. A control injection with the organism was made in the opposite flank.

In many animals which showed a strong reaction to egg protein, there appeared a large red lesion which spread to the margins of the reacting area but never beyond. The control sites were always small red papules or pustules which never attained a comparable size and healed rapidly. (See Table IV.)

The importance of this is obvious in explaining the development and persistence of local lesions in individuals having a high humoral im-

munity. On the one hand the cells locally may become so altered that the immune bodies of the blood do not penetrate into the lesion or, on the other hand, the cells are so injured that the immune bodies no longer exert the usual protecting mechanism. In animals which did not react strongly to the egg there was quite the opposite effect. The lesions were actually smaller than in the controls and healed more rapidly.

TABLE V

Infection by B. lepi-septicum of Areas of Skin Reacting to Egg into Which Bacterial Immune Serum Was Injected Mixed with the Egg Antigen

Rabbit number	Amount of egg protein in skin	Amount of serum mixed with egg	Intensity of skin reactivity to egg serum mixture (after 24 hours)	Infection in egg reacting site (after 24 hours)	Control infection into normal skin of animal (after 24 hours)
	cc.	cc.			
4-28	0.3	1	Marked	Mod. 6.5 x 5.5 cm.	Small 2.5 x 2.5 cm.
4-30	0.3	0.2	Mod. oedema	Mod. 4 x 3.5 cm.	Small 1 x 1 cm.
4-22	0.3	2	Marked oedema	Large necrotic 7 x 5 cm.	Small 2.5 x 1.5 cm.
4-33	0.3	0.2	Slight	Small 1 x 1 cm.	Small 1.5 x 1.5 cm.
4-25	0.3	0.4	Marked oedema	Large 9.5 x 5 cm.	Small 2.5 x 2.5 cm.
4-44	0.3	4	Marked	Large 8 x 5 cm.	Mod. 3 x 2.5 cm.

Controls Using Normal Serum Mixed with Egg

4-29	0.3	0.1	Marked	Large necrotic 7.5 x 5 cm.	Small 2.5 x 2 cm.
4-35	0.3	0.2	Slight	Small 2.5 x 2.5 cm.	Small 1 x 1 cm.
4-24	0.3	0.2	Moderate	Large 8 x 5.5 cm.	Small 2.5 x 2.5 cm.
4-26	0.3	0.4	Moderate	Large 6.5 x 5.5 cm.	Small 1.5 x 1 cm.

II. Infection of Egg-Reacting Sites into Which Immune Serum Is Injected Simultaneously with the Egg Antigen

Normal rabbits sensitized to egg were given intradermally 0.3 cc. of egg solution mixed with varying amounts of immune serum of recognized potency. 24 hours later a large reacting area was found which was identical to that produced by egg alone, though perhaps somewhat larger and redder. When such an area was injected with *B. lepi-septicum* the spread was just as rapid and necrosis just as extensive as in the animals receiving egg alone, or in the controls receiving normal serum mixed with the egg. (See Fig. 2 and Table V.)

From this experiment it is obvious that either, (1) the immune serum has diffused out of the lesion or, (2) has been denatured in some way or, (3) the cells have been so injured that they no longer profit by its presence. The latter hypothesis can be excluded by the next experiment.

III. Infection of Egg-Reacting Sites When Immune Serum and Organisms Are Injected Simultaneously

Normal rabbits were sensitized to egg as outlined above. Reacting areas were produced by injecting 0.3 cc. of egg intradermally. These wheals were injected with *B. lepi-septicum* with which was mixed 0.1 or 0.2 cc. of immune serum. The local protection was striking. No lesion developed in the region of the injection, but when the dose of serum was small and the infecting dose of organisms large, the infection appeared at the periphery of the egg-reacting site where there were necrosis and other manifestations of infection. (See Fig. 3.)

It is evident that immune serum, if present, is a potent protective agent, even in the presence of injured cells. This serum seems more accessible to the injured cells of this type of lesion when injected into the tissues than when supplied through the general circulation. Normal serum has no effect when injected with the bacteria.

IV. Infection of Egg-Reacting Sites Which Are Induced by Injecting Bacterial Immune Serum with the Egg Antigen and in Which Normal Serum Is Injected with the Infecting Organisms

It seemed conceivable that the immune serum present during the antigen antibody reaction might be altered but not destroyed, and the following experiment has been devised to learn whether the immune serum could be reactivated by normal serum which itself has no protective value.

Normal rabbits were sensitized to egg and the usual hypersensitive reaction was induced by injecting intradermally 0.2 cc. of egg solution mixed with 0.5 cc. of immune serum on each side of the animal. 24 hours later the areas were injected with *B. lepi-septicum* and, in addition, normal serum was injected into the reacting area, sometimes into the same site as the organisms, sometimes in another part of the wheal. The other side was infected with an equal dose of organisms but no normal serum was added. There was striking protection in the area receiving the normal serum, while the area receiving only bacteria showed the usual fulminating lesion.

TABLE VI

Wheals Induced in Both Flanks of Egg-Sensitized Rabbits by Injecting a Mixture of Egg and Bacterial Immune Serum. Injected 24 Hours Later, Injecting at Time of Infection Normal Serum in One Wheel, Healed Serum in Other

Number of animals	Amounts of egg and immune serum in mixture	Wheel receiving fresh normal rabbit serum at time of infection			Control wheel receiving only heated normal rabbit serum, or no serum at time of infection		
		Severity of preliminary wheal	Amount of normal serum injected	Result of infection	Severity of preliminary wheal	Substance injected with infection	Result of infection
4-48	0.3 cc. egg 0.4 cc. immune serum	Moderately severe	0.3	Small healing lesion, 2 x 2.5 cm.	Moderately severe	Nothing	Large spreading lesion, 8.5 x 6 cm. Necrosis at dependent part
4-57	0.3 cc. egg 0.6 cc. immune serum	Moderate reaction	0.4	Lesion moderate size, 7 x 3.5 cm. Healed promptly	Moderate reaction	Nothing	Large spreading necrotic lesion, 8.5 x 7 cm.
4-55	0.2 cc. egg 0.4 cc. immune serum	Moderate reaction	0.4	Moderate healing lesion, 8 x 4 cm.	Moderate reaction	Nothing	Large spreading necrotic lesion, 4 x 7 cm.
4-58	0.3 cc. egg 0.6 cc. immune serum	Moderate reaction	0.4	Small healing lesion, 3 x 3 cm.	Moderate reaction	Nothing	Large spreading necrotic lesion, 8.5 x 6.5 cm.
4-71	0.2 cc. egg 0.4 cc. immune serum	Moderate reaction	0.2	Moderate size, flat, sl. necrosis, healing, 5.5 x 5 cm.	Moderate reaction	0.2 cc. heated rabbit serum	Large spreading necrotic lesion, 9 x 6 cm.
4-74	0.2 cc. egg 0.4 cc. immune serum	Moderate reaction	0.4	Pale, healing, 4 x 4 cm.	Moderate reaction	0.4 cc. heated rabbit serum	Large spreading necrotic lesion, 9.5 x 5 cm.
4-67	0.2 cc. egg 0.4 cc. immune serum	Severe hemorrhagic	0.4 (Guinea-pig serum)	Small, pale, healing, 3.5 x 3.5 cm.	Moderate reaction	0.4 cc. heated guinea-pig serum	Large spreading necrotic lesion, 11.5 x 4 cm.

This experiment indicates the reactivation of the inert immune serum by normal serum. Experiments in a small series of animals indicate that the restoration of activity to the immune serum is due to the addition of *complement* or some other thermolabile substance.

Animals sensitized to egg were injected with 0.2 cc. of egg and 0.4 cc. of immune serum into the skin of both flanks. 24 hours later the wheals on each side were infected with equal doses of *B. lepi-septicum*. At the same time 0.2 cc. to 0.4 cc. of fresh *normal* rabbit or guinea-pig serum was injected into one wheal while a similar amount of the same serum *heated* at 60° for 20 minutes was injected into the opposite wheal. Within 24 hours the wheal receiving the heated normal serum showed an extensive necrotic infection, while the wheal receiving the unheated normal serum showed definite evidence of healing. (See Table VI and Fig. 4.)

Further study is being made of the nature of this phenomenon and the indications are that some complement-like substance is used up in the egg-antibody reaction. Such a substance is probably necessary for the effective utilization by the cells of the immune serum which is present in the lesion but is inert.

DISCUSSION

It has been demonstrated in the preceding experiments that extensive tissue injury, due either to chemicals or to an intense antigen-antibody reaction allows pathogenic bacteria to disseminate with remarkable rapidity. This is observed even in animals with high humoral immunity though in such animals infection does not usually extend beyond the injured tissues. Such a mechanism of previous injury may be of importance in explaining the rapid development and persistence of large, local infections even in the presence of a high degree of humoral immunity.

Certain of the actions of immune serum in focal infection seem clear from these experiments. When preliminary injury is intense the tissues involved seem to become segregated from the body as a whole, so that circulating immune substances do not reach the cells of this portion in spite of the marked hyperaemia. In areas thus isolated pathogenic organisms may thrive and destroy the unprotected tissues. However, the cells outside, abundantly supplied with circulating antibodies, withstand the processes of infection as do the wandering cells

entering the lesion. Hence, in the immune animal the lesion fails to spread, exudate from it is more purulent in character and repair proceeds more efficiently. On the other hand when the local damage is slight, no such tissue segregation occurs. The supply of immune substances may be actually increased by the irritant. In such cases healing begins almost at once.

In the type of injury produced by a local antigen-antibody reaction, a segregation of the area is also striking. But an additional factor is introduced which lowers resistance, for bacterial immune serum present during the reaction fails to exert a protective influence unless unheated complement is added. This using up of a complementary substance perhaps demonstrates a mechanism by which specific immune processes may be disturbed in disease. In addition, one would not expect the administration of therapeutic serum intravenously to be effective against such a segregated lesion.

In the absence of specific immune substances, it is important to the host to restrict the invading organisms to a localized area. It has long been assumed that the allergic animal is better equipped with such a localizing mechanism. In Table I it is seen that strong reactors withstand infection better than weak reactors. If, however, a large lesion develops the animal quickly loses its skin reactivity and the infection spreads just as actively as in the weak reactor. Furthermore, the allergic reaction may be so severe and the local damage so great that the inflammatory zone becomes analagous to the wheal of an antigen-antibody reaction and the infection instead of being restrained spreads with great rapidity.

It must also be emphasized that allergy is not the only tissue factor to be considered in localizing infections. Our observations on *B. leptisepticum* quite confirm Rich's (10) findings with the tubercle bacillus that localization of the lesion often depends upon an intrinsic immunity of the cells against the injurious effects of infection. This may be easily demonstrated by the fact that a temporarily desensitized allergic animal without demonstrable humoral immunity may possess the capacity to restrain the spread of an infecting organism.

In spite of these variations in the influence of allergy on the course of infection our observations lead us to the belief that allergy is more than a "concomitant phenomenon" of the tissue immune state, and a

number of facts regarding the tissue reactions in an allergic animal can be cited which tend to confirm this view.

1. It has been shown that mild irritants such as broth filtrates when injected several hours previous to infection tend to protect locally even in the absence of general immunity. This preliminary inflammation is seldom comparable in extent and intensity, or in the amount of oedema observed in the wheals produced by egg in the sensitized animal, or in the lesion produced by chemical irritants.

2. Within a few minutes after the introduction of a mild irritant into the skin of a strong reactor the endothelial cells of the area become highly permeable on the *vessel lumen side* as is illustrated by injecting India ink intravenously during the first few minutes of the reaction and noting the phagocytosis of ink by the vascular endothelium. Negative reactors practically never show this phenomenon (11).

3. A few hours after injection into the skin of a bacterial filtrate to which the animal responds, the endothelial cells undergo a peculiar alteration so that if homologous filtrates or certain heterologous bacterial filtrates are injected intravenously, a hemorrhagic necrosis occurs at the site of skin injection. After an animal develops humoral immunity this phenomenon no longer takes place (12, 13). If, however, the homologous filtrate is injected into the site of the skin test no hemorrhagic necrosis occurs. On the contrary, an accelerated skin reaction develops in which redness and swelling appears within a few hours and begins fading many hours before a control test in a normal area of skin.

These observations make us believe that the endothelial cells of a strong reactor are exceedingly sensitive to products of certain bacteria and that within a relatively short time they become, on the tissue side, impervious to these products, perhaps through some protoplasmic alteration. Such stimulated cells are not readily destroyed by the products of infection present in the tissues but may be seriously changed if such products are absorbed and reach them through the medium of the vascular system which would be analogous to the hemorrhagic necrosis following the intravenous injection of filtrate. Clinically such lesions are not uncommon during the course of severe infections.

CONCLUSIONS

1. Animals showing natural bacterial allergy to filtrates of *B. lepi-septicum* survive infection by this organism more frequently than weak reactors. This increased resistance is manifested by better localization of infection.

2. Bacterial filtrates injected into skin 24 hours before infection exert a non-specific protection of that area against the organism, even

in susceptible animals. The cells of this protected area seldom undergo necrosis when infected.

3. Severe injury of tissues either by chemicals or an antigen-antibody reaction produces a loss of local resistance even in immune animals. Mild injuries have the opposite effect. It is believed that in cases of severe injury, the affected areas undergo a segregation from the circulating antibodies.

4. When bacterial immune serum is injected with a protein antigen into the skin of a sensitized animal, a local alteration occurs in which substances necessary for the effective action of the immune serum are destroyed.

5. A protective action is restored to the altered immune serum by addition of complement to the lesion.

6. It is felt that allergy is not the chief mechanism in cellular resistance to infection, however data are advanced which suggest that allergy does exert local protection by acceleration of the immune processes and by rendering the cells locally refractory to further injury.

7. Chronic infection by a single strain of organism excites cellular reactivity to many strains of bacteria often unrelated biologically. Hence a non-specific mechanism for localizing infections throughout the body may be induced.

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EXPLANATION OF PLATES

PLATE 18

FIG. 1. No. 4-26. Animal sensitized to egg. Wheal induced with 0.3 cc. egg protein 24 hours before infection in it and in a control site of normal skin. Drawing, made 24 hours after infection, shows relative size of lesion.

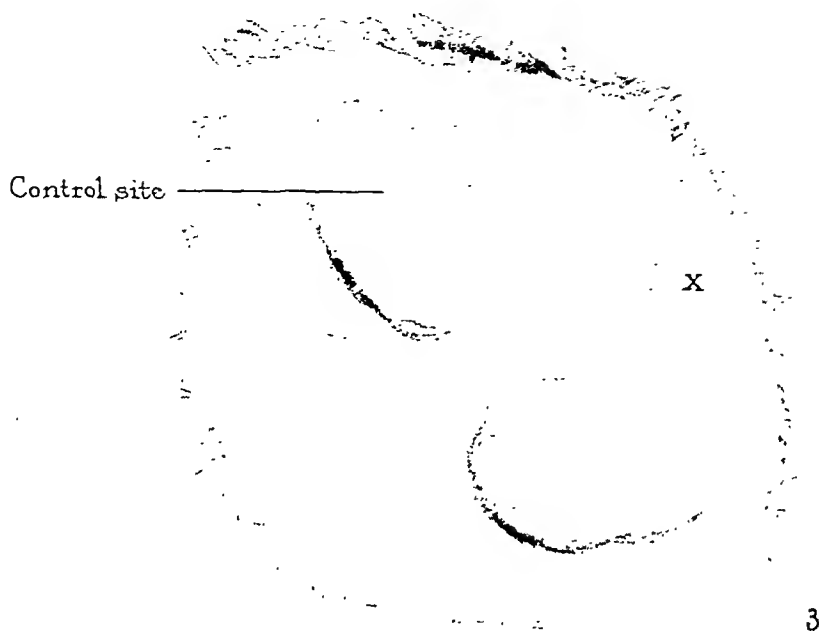
FIG. 2. No. 4-25. Animal sensitized to egg. Wheal induced by injecting a mixture composed of 0.3 cc. of egg protein and 0.4 cc. *B. lepi-septicum* immune serum. Infected 24 hours later. Drawing, made 24 hours after infection, shows huge infection in spite of immune serum.

PLATE 19

FIG. 3. No. 4-27. Wheal induced as in Fig. 1. *B. lepi-septicum* immune serum 0.2 cc. injected into wheal at X with infecting bacteria. Drawing, made 24 hours after infection, shows striking protection of the portion of the wheal receiving immune serum.

FIG. 4. No. 4-74, May 24, 1930. Wheals induced in both flanks by injecting 0.2 cc. egg and 0.04 cc. bacterial immune serum in sensitized animal. Infected 24 hours later with equal dose of bacteria, but in one site 0.4 cc. normal rabbit serum was injected with organisms; in the other an equal amount of rabbit serum heated 60° for 20 minutes.





HOST FACTORS IN THE PATHOGENESIS OF PNEUMOCOCCUS PNEUMONIA

I. THE REACTION OF THE LUNGS TO PNEUMOCOCCUS AUTOLYSATE IN SENSITIZED RABBITS

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PLATE 20

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Allergy or hypersensitiveness to bacterial products is believed to be an important factor in modifying the reaction of the host to many infectious diseases. The localization of the antigen by a prompt inflammatory response in the allergic host has been considered by some as evidence that allergy is an expression of partial immunity, by others that it is one of increased susceptibility. To what extent this phenomenon may play a part in the pathogenesis and clinical course of pneumococcus pneumonia in man is unknown but has seemed worthy of study. The present investigation was consequently undertaken to observe the response of the lung to pneumococcus antigen in animals previously rendered hypersensitive to the pneumococcus. No attempt has been made to answer the question whether the allergic state is beneficial or harmful.

The existence of allergy in man to pneumococcus is suggested by the local reactions obtained in many human beings when various fractions or products of the pneumococcus are injected intradermally (1, 2, 3, 4, 5, 6, 7), and by the rapid development of the pathological lesion of pneumococcus pneumonia with its characteristic diffuse exudate. Lauche (8), in fact, largely on clinical and theoretical grounds, has strongly advocated the view that hypersensitiveness plays an important rôle in the pathogenesis of lobar pneumonia, though presenting no direct experimental observations in support of this conception.

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In contrast with man, young rabbits are rarely skin reactive to pneumococcus antigen and the intratracheal inoculation of virulent pneumococci in them usually produces a septicemia without pulmonary lesions resembling those found in human pneumonia. It seems logical, therefore, that the question has been raised whether the somewhat peculiar susceptibility of man to pulmonary infection by the pneumococcus and his capacity to localize the infection in the lung are related to allergy to that bacterium or its products and, if so, whether these characteristics are paralleled by cutaneous sensitivity.

Although experiments on the relationship of general allergy to pulmonary reaction have been reported by Schlecht and Swenker (9), who first sensitized guinea pigs to human serum by intraperitoneal inoculation and later caused the animals to inhale the same antigen with a resultant exudative inflammation of the lungs, comparable experiments with pneumococcus as an antigen have not been found in the literature. Wadsworth (10), however, has reported experiments that are highly suggestive. He immunized rabbits with bile and saline solutions of pneumococci and 2 weeks later was able to produce a diffuse inflammatory response resembling the pneumonia of man by injecting a virulent culture intratracheally. In repeating this work Kirkbride (11) assumed, but did not demonstrate the existence of sensitivity at the time of the intratracheal injection and, furthermore, she found the pathological picture confused by pulmonary infections caused by *B. lepi-septicum* or *B. bronchisepticus*. She concluded "that the development of the diffuse exudative reaction in the lung is not due to an acquired hypersusceptibility but to intrinsic qualities possessed by the pneumococcus itself."

Experimental Procedure

Some of the conditions found in human beings can be simulated experimentally in animals by first making animals skin sensitive to pneumococcus antigen and then introducing the same antigen into the lungs of the sensitized animals. Inasmuch as the histological picture produced locally in a sensitized skin by the inoculation of antigen is accepted to be a true inflammatory process, one might expect the capillary bed of the lungs in the sensitized animal to respond as the capillary vessels of its skin, namely, by the outpouring of an exudate. In addition, if an inflammatory condition in the lungs could be produced by a sterile isotonic solution of pneumococci, harmless to normal non-sensitive animals, the result would appear to be of more significance than if living organisms were used, since many of the experimental difficulties associated with the use of whole organisms, whether living or dead, would be avoided. Consequently rabbits were sensitized to pneumococcus by several methods and their degree of

sensitivity determined at frequent intervals by intracutaneous injections of pneumococcus autolysates. At various intervals ranging from 15 to 62 days after the first sensitizing injection an intratracheal injection of autolysate was given to each rabbit. Twenty-four hours later the animal was killed and the lungs were examined for evidence of inflammation. Young white rabbits weighing between 700 and 1200 gm. were employed.

Preparation of Autolysates.—Saline autolysates were chosen because it has been shown (12) that they cause a more clear-cut reaction than a solution of pneumococci in bile or the whole bacteria, because the use of whole bacteria for lung injections might confuse the pathological picture by evoking a foreign body response, and because the errors of time and quantity attendant upon lysis *in vivo* could be eliminated.

Type II pneumococci of known virulence were used in preparing the autolysates. In most instances an avirulent strain which had been derived from a virulent Type II strain by growth in homologous immune serum was employed. This attenuated Type II pneumococcus was an intermediate between the true virulent S form and the avirulent R form, since it grew in small smooth colonies on blood agar but nevertheless showed agglutination reactions similar to those of rough pneumococci. In the concentrated autolysates prepared from it, the type specific soluble substance was demonstrated to be present by the precipitin reaction, but only in very small amount. In a few instances a highly virulent smooth strain was employed. The pneumococci were grown in buffered, 0.5 per cent dextrose, meat infusion broth for 6 to 7 hours at 37°C. The growth was then centrifuged and washed twice with saline. The bacterial sediment from 100 cc. of broth culture was suspended in 1 cc. of saline and was allowed to autolyze aerobically at 37°C. for 5 days. At the end of this period the tubes contained a milky material, which was sterile. Gram's strain showed only Gram negative amorphous debris. The pH of these solutions ranged from 5.4 to 5.8. Tenth normal sodium hydroxide was added to obtain a pH of 7.2 to 7.4. The whole mixture was centrifuged until the supernatant fluid became water clear, and the latter was then bottled without preservatives. Chemical tests on this solution showed a positive Biuret and a faint cloud with trichloroacetic acid. The presence of pneumococcus protein and the type specific polysaccharide was demonstrated by the precipitin test (13).

With the autolysates made from either the avirulent or virulent Type II pneumococcus, none of the primary toxic skin reactions were observed. The hemotoxin described by Cole (14), and the necrosis producing factor described by Parker (15), had probably been eliminated by the 5 day period of autolysis at 37°C. under aerobic conditions. Nevertheless, in order to avoid any possibility of error arising from the presence of these toxic products, the autolysates were heated at 60°C. for 30 minutes, since it has been shown that the hemotoxin and the necrotizing

fraction are thermolabile. The purpura producing principle described by Julianelle and Reimann (16) may possibly have been present, but since no purpuric lesions were noted in the skin or lungs of non-sensitized rabbits and none in the lungs of sensitive rabbits there is no reason to suppose that, if present, it played any part in the results.

Standardization of Autolysates.—The autolysates were standardized on the basis of total nitrogen content, which varied from 150 mg. to 34 mg. per 100 cc. in different lots. Preliminary intradermal tests with an autolysate made from the avirulent pneumococcus and containing 125 mg. of nitrogen per 100 cc. gave a satisfactory range of intensity of skin responses from intradermal injections of 0.2 cc. (0.25 mg. N). Consequently that volume of each autolysate which contained 0.25 mg. of nitrogen was fixed as the unit skin test dose. It was found, however, that autolysates prepared from the virulent S strain of Type II pneumococcus and containing large amounts of soluble polysaccharide, required slightly less volume to elicit a reaction like the response from the standard injection unit than would have been expected on the basis of nitrogen content. It was inferred from this that some of the local inflammatory response might be caused by the specific polysaccharide. This factor in the case of autolysates prepared from virulent pneumococci was controlled by using a volume which was shown by actual inoculation tests to cause the same intensity of skin reaction as that caused by the standard dose of autolysate prepared from the avirulent organism.

Methods of Sensitization.—Various methods of sensitization were used and the animals are grouped accordingly. The details showing the number and spacing of injections for each rabbit are presented in Table I.

Group 1. Three rabbits (Nos. 6A, 7A, and 10A) received only intradermal injections of 0.2 cc. of avirulent Type II autolysate every third or fourth day for varying periods before intratracheal injection. These injections also served for the tests of cutaneous sensitivity.

Group 2. Five rabbits (Nos. 1B, 4B, 5B, 12B, and 14B) received intravenous injections of 0.2 cc. of avirulent Type II autolysate every 3 or 4 days for varying periods and in addition 0.2 cc. of the same autolysate intradermally for skin tests 24 hours before each intravenous injection.

Group 3. Four rabbits (Nos. 4C, 7C, 8C, and 11C) were treated with whole avirulent intermediate Type II pneumococci. Each received a single subcutaneous injection of killed organisms, the dose in each instance being the growth from 50 cc. of a 6 hour 0.5 per cent dextrose infusion broth culture. In addition, Nos. 4C and 8C each received three intradermal skin-test injections of avirulent Type II autolysate, No. 7C six intradermal tests and No. 11C, five.

Group 4. Two rabbits received intravenous injection of avirulent Type II pneumococci. Rabbit 5C was given four injections, the first being of killed organisms, the other three of live organisms. The doses were the growth from 5 cc., 5 cc., 7 cc. and 7 cc., respectively of a 6 hour 0.5 per cent dextrose, infusion broth culture. Rabbit 4E received two injections of killed bacteria, each dose

being the growth from 10 cc. of broth culture. In addition No. 5C received seven intradermal tests with avirulent Type II autolysate, No. 4E, six.

Group 5. Four rabbits (Nos. 3F, 5F, 2G, and 6G) were sensitized by a single intravenous injection of killed virulent Type II pneumococci, the dose in each case being the growth from 5 cc. of broth culture. They were skin tested with autolysate at 3 to 5 day intervals over a period of 43 days in the case of No. 3F, 51 days in the case of No. 5F, 43 days in the case of No. 2G, and 54 days in the case of No. 6G.

Group 6. Two rabbits (Nos. 1K and 2K) were sensitized, each by a single intravenous injection of killed avirulent, rough, Type I pneumococci. The sensitizing dose consisted of the growth from 5 cc. of broth culture. Skin tests with avirulent Type II autolysate were done at 3 to 5 day intervals over a period of 45 days in each case.

Skin Tests.—The skin tests for sensitivity to pneumococcus were made by intradermal injection of the standard dose of autolysate in the skin of the flanks which had been shaved 48 hours previously. The days on which the skin tests were made and the degree of reaction exhibited at each test are shown for each rabbit in Table I. The reactions in sensitive animals appeared in 4 to 8 hours. Positive reactions consisted of local areas of erythema and edema which increased to a maximum size in 18 to 24 hours. Neither induration nor nodule formation nor the "secondary reaction" of Andrewes, Derick and Swift (17) were seen. Four degrees of intensity were recorded:

1. Slight (\pm). Erythema faint but definite. Edema very scanty or absent. Measurements taken in the short and long diameters showed extreme readings of 1 x 1 cm. and 6 x 2 cm.

2. Moderate (+). Erythema deeper. Definite edema present. Lesions on the average somewhat larger than in 1. Size varied between 1.5 x 2 cm. and 6 x 3 cm., the average being 2 x 3 cm.

3. Marked ($\pm\pm$). Erythema quite intense. Edema considerable. Average size about 3 x 4 cm.

4. Maximum (++). Very intense erythema, sometimes purpuric. Edema extensive with sagging skin. The lesions often required 72 hours to subside and superficial desquamation of the skin resulted frequently. No ulceration.

Although no gross necrosis with ulcer formation was noted, it is conceivable that the extreme reaction of tissue injury which has been reported by others (12) might have resulted from the use of a more concentrated autolysate. The skin test unit which was adopted for this study permitted a comparison of degrees of reactivity, and for that reason was considered most satisfactory.

Intratracheal Injections.—Twenty sensitized rabbits and seven non-sensitive normal controls were injected intratracheally with clear sterile isotonic pneumococcus Type II autolysates of standard nitrogen value. As shown in Table I, the injections were always made the day following a skin test in order to correlate the results as nearly as possible with the just determined skin sensitiveness of the

animal. The rabbits were prepared by shaving the neck and sterilizing the skin. A hypodermic needle was inserted between the rings of the tracheal cartilages and the autolysate was injected slowly while the animal was held with the fore part of the body slightly elevated. After completion of the injection the animal was kept with fore part elevated for 2 minutes. A standard volume of 5 cc. of saline was

Cutaneous Reactivity and Pulmonary Reaction

Group	Method of sensitization	Rabbit No.	Initial sk. test		Weeks 1							2								3							4				
			Days	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25			
1	Avirulent pneumococcus Type II autolysate intradermally	6A 7A 10A	- - -	- - -	- - -	- - -	- - -	- - -	- - -	- - -	- - -	- - -	- - -	- - -	- - -	- - -	- - -	- - -	- - -	- - -	- - -	- - -	- - -	- - -	- - -	- - -	- - -	- - -			
2	Avirulent pneumococcus Type II autolysate intravenously	1B 4B 5B 12B 14B	- # - # #	s s s s s	- # # # #	- # # # #	- # # # #	- # # # #	- # # # #	- # # # #	- # # # #	- # # # #	- # # # #	- # # # #	- # # # #	- # # # #	- # # # #	- # # # #	- # # # #	- # # # #	- # # # #	- # # # #	- # # # #	- # # # #	- # # # #	- # # # #	- # # # #	- # # # #			
3	Killed avirulent pneumococcus Type II subcutaneously	4C 7C 8C 11C	- - + -	s s s s	- - - -	- - - -	- - - -	- - - -	- - - -	- - - -	- - - -	- - - -	- - - -	- - - -	- - - -	- - - -	- - - -	- - - -	- - - -	- - - -	- - - -	- - - -	- - - -	- - - -	- - - -	- - - -	- - - -				
4	Avirulent Type II pneumococci intravenously	5C 4E	- +	s s	- +	- +	- +	- +	- +	- +	- +	- +	- +	- +	- +	- +	- +	- +	- +	- +	- +	- +	- +	- +	- +	- +	- +	- +			
5	Killed virulent Type II pneumococci intravenously	3F 5F 2G 6G	- # + #	s s s s	- # + #	- # + #	- # + #	- # + #	- # + #	- # + #	- # + #	- # + #	- # + #	- # + #	- # + #	- # + #	- # + #	- # + #	- # + #	- # + #	- # + #	- # + #	- # + #	- # + #	- # + #	- # + #	- # + #				
6	Killed Type I-R pneumococci intravenously	1K 2K	- +	s s	- +	- +	- +	- +	- +	- +	- +	- +	- +	- +	- +	- +	- +	- +	- +	- +	- +	- +	- +	- +	- +	- +	- +				

s = sensitizing injection; t = intratracheal injection of avirulent pneumococcus Type II autolysate.
 * - to ++ signs following intratracheal injection indicate degree of acute exudative inflammation.
 - = negative; ± = slight; + = moderate; ± ± = marked; ++ = maximum.

used and each dose contained 1 to 1.5 mg. of total nitrogen. Except for a variable amount of coughing, no symptoms were shown by the rabbits.

Examination of the Lungs.—The animals were killed 24 hours after intratracheal injection by inserting a needle through the cisterna magna into the base of the brain. This procedure was rapid and avoided the errors of technique which

etherization or air embolus entail. The chest was opened aseptically. One half of the right lung was excised and cultured in broth and on a blood agar plate. An injection of Zenker's formol solution was made *in situ* to preserve the contour and to prevent atelectasis. Histological sections were prepared and stained with hemotoxylin and eosin, Mallory's connective tissue stain, and MacCallum's

Staphylococcus Aureolysate in Sensitized Rabbits

5					6							7							8							9							
30	31	32	33	34	35	36	37	38	39	40	41	42	43	44	45	46	47	48	49	50	51	52	53	54	55	56	57	58	59	60	61	62	63
1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
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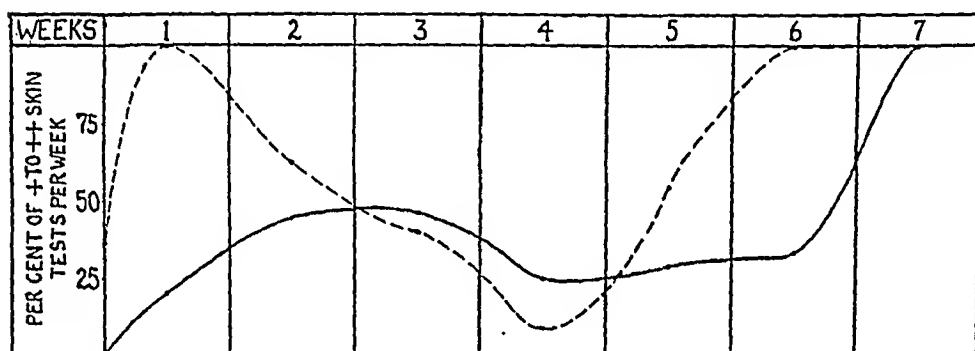
of skin tests and degree of cutaneous sensitivity are indicated by - to \div signs.

modification of Goodpasture's bacterial stain. Grossly, no significant changes were observed on the surfaces, and sections of the fresh lung were not made. The cultures were sterile except in three cases. In two rabbits, Nos. 5F and 6G, whose lungs showed no acute inflammatory reaction, *B. bronchisepticus* was found; in a third rabbit, No. 3F, whose lung showed a pleural scar, *B. leptosepticus* was present.

but the pathological picture showed an acute exudate in the alveoli which was unlike the usual response to *B. lepiæpticum*. Nevertheless, because of the presence of the bacteria, the results from this rabbit may be of doubtful significance.

Experimental Results

Cutaneous Sensitivity.—The course and degree of cutaneous sensitiveness to pneumococcus autolysate exhibited by each rabbit are shown in Table I. It will be noted in the first place that of the 20 rabbits, 9 showed no reaction on preliminary test, 7 showed a slight (\pm) reaction, and 4 showed a moderate (+) reaction; in the second place, that all but two rabbits (Nos. 6A and 8C*) exhibited moderate



TEXT-FIG. 1. The effect of existing hypersensitiveness on the course of cutaneous sensitivity to pneumococcus autolysate. Unbroken line, initially non-sensitive rabbits; broken line, initially sensitive rabbits.

to very marked cutaneous reactivity at some time subsequently during the course of the experiment; in the third place, that the course of the sensitiveness in general was wavelike in character with a markedly positive phase during the first 2 to 3 weeks, a negative or only slightly positive phase beginning in the third and running through the fourth into the fifth week with the return of a strongly positive phase in the sixth and seventh weeks; and lastly, that in spite of the general trend there was marked individual variation both in the degree and the course of sensitivity irrespective of the method of sensitization. Only

* 8C was not tested during the first 20 days and would probably have reacted during this period.

one obvious factor would appear to have exerted any significant effect on the course of events, namely, the initial state of sensitivity as determined by the preliminary skin test.

This is brought out in Text-fig. 1 in which it will be seen that the originally non-sensitive rabbits responded more slowly and less markedly during the first period of increased sensitivity than the originally sensitive rabbits. Subsequent to this, however, no significant differences between the two groups are apparent. Further discussion of the course and degree of cutaneous reaction in rabbits sensitized to the pneumococcus is not pertinent here and would obviously be of no significance in view of the marked individual variation exhibited and the small group of animals studied. It will consequently be reserved for a subsequent paper in which the results obtained in a larger series of rabbits will be presented.

Pulmonary Reaction.—An acute exudative inflammation of the lungs was found in a considerable number of the sensitized rabbits following the intratracheal injection of sterile pneumococcus autolysate (Table I). Histological examination showed that the exudate consisted largely of polymorphonuclear leukocytes and serum. Fibrin was not conspicuous and there was no necrosis nor hemorrhage. The extent of the inflammation varied considerably and may be divided into four degrees, as follows: (1) slight (\pm), denoting small focal areas of inflammation consisting of a polymorphonuclear leukocytic exudate lining some of the bronchioles and a thin layer of leukocytes and serum in the adjacent alveoli; (2) moderate (+), denoting a more extensive involvement with focal areas of exudate in the bronchioles and alveoli; (3) marked ($\pm\pm$), denoting a still more extensive involvement with confluent areas of serous and cellular alveolar exudate; (4) maximum ($++$), denoting involvement of about one-quarter of the total lung with peribronchial, bronchiolar, and alveolar exudate of leukocytes and serum (Figs. 1 and 2).

The cultures of the lung made at autopsy were sterile in all but one (No. 3F) of the rabbits which showed an acute inflammation of the lungs, so that an existing infection would appear to be excluded as a cause of the acute lesion found in the sensitized animals. Furthermore, the pulmonary infections due to *B. bronchisepticus* or *B. lepi-septicum* have been shown (18) to consist of a more destructive lesion

than that encountered here. Rarely an associated fibrotic lesion of the pleura was present. Obviously a lesion of this character could not

TABLE II
Relation of Pulmonary Reaction to Cutaneous Sensitivity

Group	Rabbit No.	Cutaneous sensitivity	Pulmonary reaction	Culture of lungs
		Skin test with pneumococcus autolysate at time of intratracheal injection	Acute exudative inflammation of lungs 24 hours after intratracheal injection of pneumococcus autolysate	
1	7 normal controls	—	—	No growth
2	6A	—	—	No growth
	12B	—	—	" "
	8C	—	—	" "
3	1B	±	—	No growth
	4B	±	—	" "
	7C	±	—	" "
4	4C	+	±	No growth
5	5F	±±	—	<i>B. bronchiseptica</i>
	6G	±±	—	" "
	5C	±±	±	No growth
	11C	±±	±	" "
	7A	±±	+	" "
	5B	±±	+	" "
	2G	±±	±±	" "
6	10A	++	—	No growth
	4E†	++	±	" "
	1K	++	±	" "
	14B	++	++	" "
	2K	++	++	" "

— = no reaction; ± = slight reaction; + = moderate reaction; ±± = marked reaction; ++ = maximum reaction.

† Much of the autolysate did not reach the lungs but was found at autopsy to have been injected into the peritracheal tissues.

be considered a part of the acute response. A careful examination of the lungs of many uninoculated rabbits often showed pleural and sub-

pleural interstitial thickening which was interpreted to be the result of a previous injury. This type of lesion was easily distinguishable from the acute exudative inflammation found in the sensitized rabbits. With Mallory's connective tissue stain these lesions showed strands of fibrous tissue, a feature which was never present in the acute lesions.

Relation of Pulmonary Reaction to Cutaneous Sensitivity.—The relationship between the cutaneous sensitivity of the rabbits and their pulmonary response to pneumococcus autolysate is shown in Table II in which the animals have been regrouped according to the degree of cutaneous reactivity exhibited at the time of the intratracheal injection.

Group 1 consists of 7 non-sensitized normal controls whose skin tests with pneumococcus autolysate were negative. None of these rabbits showed any pulmonary reaction following intratracheal injection of the autolysate

Group 2 includes three rabbits which showed negative skin tests at the time of intratracheal injection. One of these, No. 12B, had previously been skin-sensitive but was in a negative phase at the time of the intratracheal injection. Another, No. 8C, was initially moderately skin sensitive but the only two subsequent tests made were negative. The third, No. 6A, had failed to become reactive at any time, although eight intradermal injections of autolysate had been given over a period of 28 days. None of these animals showed any pulmonary reactions.

Group 3 consists of three rabbits, Nos. 1B, 4B, and 7C, which showed slightly positive skin reactions at the time of intratracheal injection. None of these rabbits had become highly skin sensitive in response to the sensitizing injections and none had a detectable pulmonary reaction when killed 24 hours after intratracheal injection.

Group 4 includes but one rabbit, No. 4C. This animal showed moderate skin reactivity and a slight, but definite pulmonary response to the pneumococcus autolysate.

Group 5 includes seven rabbits which had marked cutaneous reactions at the time of intratracheal injection. All but two of these showed an acute exudative inflammation in the lungs, slight in two, moderate in two, and marked in one.

Group 6 contains five animals which showed extreme skin reactivity. Four of these showed acute inflammation in the lungs. No. 10A was negative; Nos. 4E and 1K showed slight but definite reactions; Nos. 14B and 2K showed the maximum degree of inflammation.

Rabbit 3F, although markedly skin sensitive and showing an acute exudation in the lungs has been excluded from the above tabulation because the culture yielded *B. leptiseticum*.

As may be seen from Table II, the results from intratracheal injection of pneumococcus autolysate in skin-sensitive rabbits in general parallel the degree of skin reactivity to the same antigen. In none of the 10 rabbits in which the skin was not reactive, whether non-sensitized controls or sensitized rabbits in a negative phase of cutaneous reactivity did the lungs show any pulmonary response when pneumococcus autolysate was brought into contact with the pulmonary tissues. Similarly, in none of the three slightly skin-sensitive rabbits did the lungs show any detectable inflammation. On the other hand 10 of the 13 animals showing moderate to extreme cutaneous sensitiveness showed clear-cut acute exudative inflammation in the lungs. The three negative results are not surprising in view of the mechanical factors of the experiment. Obviously, the technique of intratracheal injection lacked the fine degree of accuracy of the intradermal injections in which the dosage and the localization of the autolysate in a given skin area were quite definite. In the case of the intratracheal injections, there was no certainty that all the injected fluid reached the lungs and, granting that it did, other variables such as the greater surface area of the intra-alveolar spaces and the possible subsequent loss of antigen by coughing may have influenced the result. Because of these factors it is not unreasonable to believe that in some instances the antigen may not have been in contact with the pulmonary tissue for a sufficient period or that the concentration of antigen may have been too weak in any given area to be effective in causing an inflammatory reaction. From the above considerations, one could expect at best only an approximate parallelism between the cutaneous and pulmonary reactions.

DISCUSSION

The results of the experiments described above indicate that when a rabbit is rendered hypersensitive to the pneumococcus, the skin reaction to pneumococcus autolysate being taken as an index of the hypersensitivity of the animal, the pulmonary reaction to contact with the autolysate closely parallels the cutaneous response, being consistently negative in non-sensitive and only slightly sensitive animals, usually positive in the moderately to extremely sensitive ones. Although the parallelism so far as degree of reaction is concerned is not a strict one in

the sensitive group, a result hardly to be expected in a biological experiment of this type, the contrast between the non-sensitive and the sensitive groups is sufficiently striking to seem significant and to leave little doubt that the acute exudative inflammation of the lungs in the sensitive animals is dependent upon the allergic state of the animal rather than upon any inherently injurious substances in the autolysate.

By the use of whole pneumococcus autolysate devoid of the primary toxic principles so far described, the experiments have been circumscribed so that the results might give an uncomplicated picture of the pulmonary reaction which depends upon the allergic state alone. Whether the pulmonary reaction is due to allergy to pneumococcus nucleoprotein, to the specific carbohydrate, or to both, or even to other unidentified pneumococcus substances in the autolysate, is not elucidated, but presumably might be determined by the use of these substances in purified form.

The bearing of these experiments on the pathogenesis of pneumococcus pneumonia in man is, of course, problematical, in view of probable host differences and because live pneumococci, which possess properties not present in the autolysate, were not employed for intratracheal injection. The chief value of introducing active infection in the rabbits would have been to acquire data on the possible relationship of the allergic response to protection, but the conditions would have been so complicated that no assurance that the reaction was due to allergy would have resulted.

In spite of the difficulties inherent in the transfer of results obtained in experimental animals to conditions in man, it would nevertheless seem not improbable that the experiments reported above may have some significance in human pneumococcus pneumonia. The possibility that allergy plays an important rôle in the disease has long been surmised, and, as mentioned above, has been extensively discussed by Lauche (8) whose arguments have been summarized by Cole (19) as follows: "The facts cited by Lauche in support of this point of view are (1) that lobar pneumonia does not occur during the first 5 months of extra-uterine life; (2) that lobar pneumonia increases in frequency during the first decades until the maximum and constant figures are reached; and (3) that the occurrence of lobar pneumonia in the new-

born has only been observed if the mother is at the same time affected with pneumonia. According to this concept the new-born contract pneumonia because they have become passively sensitized from the mother. As a rule, infants do not acquire the disease during the first 5 months because they have not had an opportunity of being actively sensitized. With the widespread distribution of pneumococci, however, there are many opportunities for infection and consequent sensitization during the first decades. Lauche states that the allergic state is not a constant one. Like immunity, it is transient and varies in degree. In order to develop pneumonia the individual must be in a hypersensitive state. If this stage is passed the individual reacts normally to infection, for instance, with a coryza."

Experimental observations in man bearing on this problem consist almost entirely of studies on the cutaneous reactions of man to a variety of pneumococcus antigens, both during health and during the active and convalescent stages of pneumococcus pneumonia. A résumé of these observations (1, 2, 3, 4, 5, 6) shows, in general, that 50 to 85 per cent of normal persons have been found to react locally to an intracutaneous injection of pneumococcus antigen with the "delayed" type of skin reaction, that during the acute stage of pneumococcus pneumonia the skin is not reactive to these same antigens, and that during convalescence from the disease a large proportion of patients sooner or later develop cutaneous sensitiveness. In addition Tillett and Francis (7) have recently shown that the reactions of convalescents may be separated into two types—(a) an immediate wheal and erythema type of reaction in response to intradermal injections of the type-specific carbohydrate of the pneumococcus, first appearing at or about the time of crisis and (b) a delayed type of reaction in response to injections of pneumococcus nucleoprotein, usually appearing somewhat later in convalescence.

Any attempt to harmonize these observations in man with the theory set forth by Lauche must presuppose that the sensitive or allergic individual who contracts lobar pneumonia becomes rapidly desensitized, at least so far as the reactive capacity of his skin is concerned, during the stage of invasion and onset, presumably through the liberation of considerable amounts of pneumococcus antigen in the lungs and its absorption and distribution to the cutaneous tissues through the

circulation. Furthermore, if desensitization does occur, it would seem probable that it is a specific desensitization to the pneumococcus rather than a general non-specific loss of cutaneous reactivity since Poole, Bumstead and Blake (20) have found that patients acutely ill with lobar pneumonia, though failing to react to pneumococcus autolysates, nevertheless frequently give a positive "delayed" type of reaction in response to an intradermal injection of a soluble antigen prepared from *B. influenzae* similar to that exhibited by many normal individuals.

While the theory discussed above may seem plausible, it cannot be accepted as fact at present because an important link in the chain of evidence is lacking, namely, observations in man on his sensitivity to pneumococcus just prior to the onset of pneumonia. It is at this point that the experiments in rabbits here reported may have an important bearing on the problem, since a hypersensitive or allergic state in the rabbit was apparently a prerequisite for an acute inflammatory response of the lung to a pneumococcus autolysate, harmless in itself in the normal or non-sensitive animal. The analogy between the conditions in lobar pneumonia in man and those in the experimental animals is, however, far from perfect, and the actual state of man's sensitiveness to the pneumococcus prior to the onset of pneumonia must be determined before the problem can be further elucidated.

SUMMARY AND CONCLUSIONS

The present study was undertaken in order to determine whether animals exhibiting cutaneous hypersensitiveness to pneumococcus would show an acute inflammatory reaction in the lungs when pneumococcus autolysate was brought into contact with the pulmonary tissues and, if so, whether the pulmonary reaction might be shown to be due to the allergic state of the animal, rather than to intrinsic properties of the autolysate. Twenty young rabbits were sensitized to pneumococcus by various procedures and their degree of hypersensitiveness determined at frequent intervals over varying periods of time by means of intracutaneous injections of pneumococcus autolysate standardized on the basis of nitrogen content and so treated as to be devoid of the known toxic principles. Twenty-four hours after the last skin test each rabbit was injected intratracheally with the same pneumococcus autolysate. Seven non-sensitive controls were simi-

larly injected intratracheally. Twenty-four hours after intratracheal injection the rabbits were killed. The lungs were removed, a portion was cultured, and the rest was examined histologically. Of the twenty sensitized rabbits, three that showed no cutaneous sensitivity and three that were only slightly skin-sensitive at the time of intratracheal injection exhibited no detectable pulmonary reaction to the autolysate; eleven of fourteen that showed moderate to extreme cutaneous hypersensitiveness were found to have an acute exudative inflammation of the lungs. The exudate consisted largely of polymorphonuclear leukocytes and serum. It varied in extent from a slight focal exudate lining the bronchioles and adjacent alveoli to a very marked diffuse involvement of considerable portions of the lung. In all but one of the eleven the cultures of the lungs were sterile. The single animal showing a positive culture (*B. lepi-septicum*) has been excluded. None of the seven non-sensitive controls showed any pulmonary reaction to the autolysate.

From these results it may be concluded that there is in rabbits a fairly close parallelism between cutaneous and pulmonary hypersensitiveness to pneumococcus autolysate and that the inflammatory response of the pulmonary tissue resulting from contact with the autolysate depends upon the allergic state of the animal rather than upon inherently injurious substances in the autolysate. The observations are in harmony with the theory that allergy may play a part in the pathogenesis of pneumococcus pneumonia in man.

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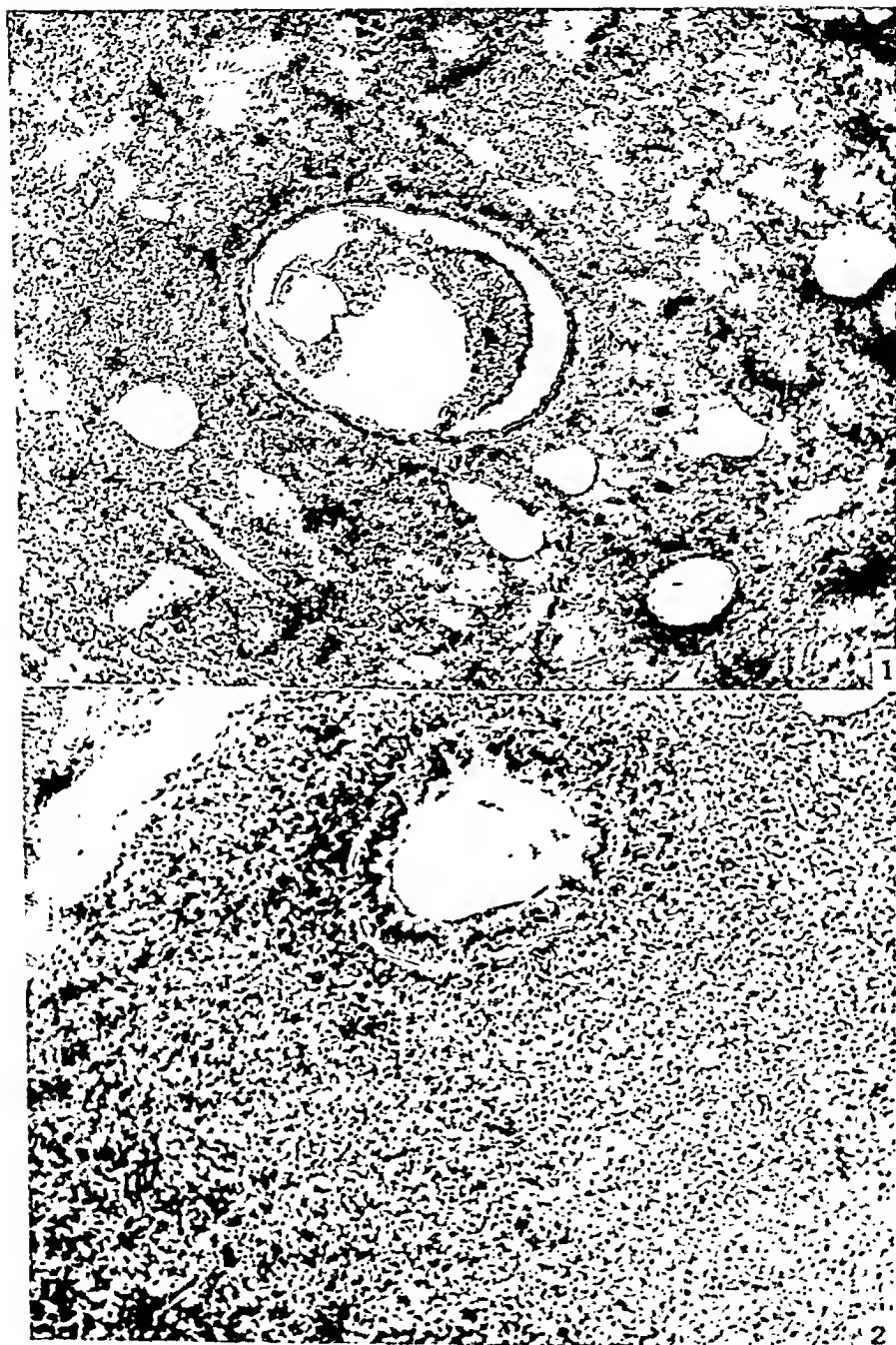
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EXPLANATION OF PLATE 20

FIGS. 1 and 2. Rabbit 2K. Acute exudative inflammation of lungs 24 hours
post-intratracheal injection of pneumococcus autolysate.





STUDIES ON A-AVITAMINOSIS IN CHICKENS

I. LESIONS OF THE RESPIRATORY TRACT AND THEIR RELATION TO SOME INFECTIOUS DISEASES

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PLATES 21 TO 23

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The diseased condition of chickens resulting from a lack of vitamin A in the diet is probably the most important of the nutritional diseases caused by A-avitaminosis in domestic animals. As pointed out by Beach (1) and Seifried and Schaaf (2), it causes serious losses on poultry farms. The clinical and anatomical picture closely resembles that found in some of the most important infectious diseases of fowls, chicken pox, coryza contagiosa, and infectious bronchitis. Golblatt and Benischek (3), Tyson and Smith (4), and Wolbach and Howe (5) have described the histological changes following a lack of vitamin A in the diet in guinea pigs, rats, and in one human individual. The characteristic changes are the substitution of stratified keratinizing epithelium for normal epithelium in various parts of the respiratory tract, alimentary tract, eyes, paraocular glands and the genito-urinary tract. No studies have been made of the histological changes in A-avitaminosis in fowls. It is the purpose of this paper to report such studies and to compare the changes with those found in certain infectious diseases.

Material

The material used consisted of 16 cases of A-avitaminosis, of which 12 were produced experimentally and 4 were spontaneous. The diet employed for the experimental production of the disease was identical with that used by Beach and

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TABLE I

Showing Source and Kind of Material Used

Number of chicken	Food received	Age at beginning of experiment	First symptoms after	Dead after beginning of experiment	Gross lesions											Histological findings
					Eyes	Nasal fossa, cleft palate	Sinuses	Larynx	Trachea, bronchi	Cavity of the mouth of (excretory ducts of the glands)	Esophagus, crop	Proventriculus	Urates (kidney)	Urates (visceral)	Other lesions	
1	Food mixture I alone	About 3 months	days 40	30 (Killed)	+	++	+	+	+	+	-	+	+	+	-	Positive
2	"	"	40	70	+	++	+	+	+	+	+	+	+	+	Oviduc-titis	"
3	"	"	45	76	-	++	+	+	+	+	+	+	+	+	-	"
4	"	"	48	79	-	++	+	+	+	+	+	+	+	+	-	"
5	"	"	44	85	-	++	+	+	+	+	+	+	+	+	-	"
6	"	"	52	87	-	++	+	+	+	+	+	+	+	+	-	"
7	"	"	51	82	+	++	+	+	+	+	+	+	+	+	-	"
8	"	"	58	80	+	++	+	-	+	+	+	+	+	+	-	"
9	"	"	54	75	+	++	+	+	+	+	+	+	+	+	Oviduc-titis	"
10	"	"	59	77	-	++	+	+	+	+	+	-	+	+	-	"
11	"	About 6 months	-	56 (Killed)	+	+	+	+	+	+	+	+	+	+	-	"
12	"	"	-	81 (Died suddenly)	-	+	+	-	-	-	+	+	+	+	-	"

13	Spontaneous cases	Lack of vitamin A	?	?	+	+++	+++	++	++	++	++	+	+	+	+	+	Oviduc-titis	Positive
14		"	?	?	-	+	+	+	+	+	+	-	-	-	-	-	-	"
15		"	?	?	-	+	+	+	+	+	+	-	-	-	-	-	-	"
16		"	?	?	-	+	+	+	+	+	+	-	-	-	-	-	-	"
17-21	Controls	Food mixture 1 + cod liver oil + greens	6 about 3, 2 " 6 months old	-	-	-	-	-	-	-	-	-	-	-	-	-	-	Negative

* Main lesions in submaxillary and lingual glands.

by us in our earlier experiments. It consisted of 50 parts wheat bran, 50 parts wheat shorts, 50 parts ground barley, 10 parts meat scrap, 10 parts fish scrap, and 20 parts soybean meal. We used chickens about 3 months old, for according to our earlier experiments and in common with other investigators, we have observed that a much shorter period is required to produce the characteristic symptoms followed by death in young, partially grown animals, than in adults. This is brought out in the present experiments in which Chickens 11 and 12 (see Table I) that were about 3 months older than the other animals, developed symptoms much later and to a minor degree than did the younger ones. To insure the presence of vitamin D, all animals were exposed daily to sunlight or to ultraviolet light. The six controls (Nos. 17-24) received the same food mixture and in addition plenty of greens (alfalfa, cabbage) and 3 cc. of cod liver oil daily given with a dropper. All of the controls remained well, failing to show any gross pathological or histological lesions. No symptoms of rickets or of a deficiency of vitamin B were observed at any time during the experiment. Table I gives all details of the experiment together with the gross pathological findings.

Methods

Most of the tissues were fixed in 10 per cent formalin immediately after autopsy, but in a few cases Zenker's fluid was used. The tissues from the nasal passages and the sinuses were decalcified, some in 5 per cent nitric acid and some in 5 per cent formol-nitric acid. All tissues were imbedded in paraffin, and for special purposes frozen sections were made. For routine stains hematoxylin and eosin, van Gieson's stain, and Mallory's methylene blue phloxine, as modified by Davidoff (6) and McGregor (7), were used. In addition the following stains were used for special purposes: methylene blue, Gram's and Giemsa's stain,—the best results being obtained with the last, with tissues fixed in formalin as well as in Zenker's fluid, by the modification given by Wolbach,—Weigert's elastic tissue stain, Weigert's fibrin stain, fat stain with Sudan III and Scharlach R, oxydase reaction, the stains with methylgreen-pyronin and fuchsin-light green, with mucicarmine (Mayer), thionin, toluidin, cresylecht-violette, and Pasini's method with waterblue-orcein, which gave distinct results in staining the keratohyalin globules, and finally stains with ferric sulfur, calcium permanganate, Congo red (keratinization), and Hamerschmidt's stain for Guarnieri's inclusion bodies.

Gross Pathology

Nasal Passages and Sinuses.—Sagittal and gross sections through the nasal cavities and the communicating sinuses show all stages of a disturbance of the lining of the nostrils and cleft palate.

Early in the disease the turbinate bones (concha superior and inferior) are filled with seromucoid, water-clear masses, which by the application of slight pressure

can be forced out of the nostrils and cleft palate. Owing to the consistency of the exudate and the complicated structure of the nasal fossae, the vestibule becomes plugged and the exudate overflows into the paranasal sinuses, often early in the disease. On the other hand, the exudate may be forced through the cleft palate, filling up the region around the vomer with seromucoid masses. Later this exudate becomes transformed into white or slightly yellow caseous masses which are found between the turbinate bones, in the nasolacrimal duct, nasal vestibule, and sinuses, causing more or less swelling of the face (Figs. 1 and 2). This involvement of the nasal passages and the sinuses may be uni- or bilateral. If on one side, the turbinate bones and the sinuses may become so enlarged that they extend over the median line and compress the opposite nasal fossa. The characteristic eye lesions in this disease, which will be described in another paper with M. Westhues, are complicated by the blocking of the nasolacrimal duct. When the sinuses are completely filled, the bulbus of the eye, which cannot move in a ventral direction, is pressed against the os frontale and sometimes forced out in a lateral direction. The protrusion of the palate into the cavity of the mouth, often observed in virus-diphtheria, is not usually seen in A-avitaminosis. After the inflammatory products have been removed, the mucous membranes throughout the nasal passages and sinuses show a peculiar, thin, rough and dry membrane, which may also be found in the mouth and throat. Unattached masses of white caseous material are often present in the cleft palate and in the surrounding mucous membrane of the roof of the mouth.

Larynx and Trachea.—Almost regularly in A-avitaminosis of chickens marked lesions occur also in the larynx, trachea and bronchi. As shown in the table, they are found in the early as well as in the later stages of the disease.

Chickens 11 and 12 on a diet lacking vitamin A showed no gross lesions, but microscopically early changes were found in the glands of the mouth, esophagus and trachea. Those in the trachea were apparently the most marked and were probably the oldest. The lesions in the trachea have as yet received no attention, but we feel that they were of special importance as it is so difficult to distinguish them from those commonly found in roup, diphtheria, and infectious tracheitis. In addition to the lesions near the entrance of the pharynx, consisting of pustule-like patches which are due to collections of white caseous material in the mucous glands, glandula cricoarytenoidea, the pathologico-anatomical picture is characterized by a peculiar laryngitis, tracheitis, and bronchitis. The mucous membrane of the anterior end of the larynx on its ventral side and in the pointed angle which is formed by the cartilages of the larynx, often shows caseous crumbly white masses. Similar lesions are frequently found throughout the entire length of the trachea and often in the bronchi. In very early stages, however, they are much less marked and may be difficult to see. The mucous membrane is then covered with a fine

film or haze; it is dry, dull, and slightly uneven on the surface, whereas the normal membrane is moist. In other cases small nodule-like particles may be visible in or beneath the mucous membrane, especially in the upper part of the trachea. In later stages the gross lesions are much more striking and may easily be seen with the naked eye. More or less thin membranes cover the mucous membrane throughout the entire length of the trachea and the bronchi. In most cases these membranes are pulled off the underlying mucosa, thus forming a thin-walled continuous tube within the trachea and bronchi (Fig. 3), a condition that might be mistaken for infectious tracheitis. The smaller bronchi very often become completely plugged with these membranes causing bronchiectasis. In some cases the larynx shows the most marked changes while in others the trachea is more involved.

Histopathology

Larynx, Trachea, and Bronchi.—The changes in these structures are the more easily understood as the normal anatomy is less complicated than in the nasal fossae and adjoining sinuses.

The first lesion is in the columnar ciliated epithelium and is characterized by an atrophy of the cytoplasm and a loss of the cilia. Along with this protoplasmic change, the nuclei often present more or less marked karyorrhexis. The atrophying and degenerating ciliated cells hang like tufts on the basement membrane and later are pushed off and may form a pseudomembrane.

While this process is going on, there appear as islands beneath the original epithelium new cylindrical or polygonal cells either singly or in layers. These are especially marked in the trachea where they may appear as focal syncytial masses (Fig. 4). The cells evidently have their origin in the original columnar epithelium. They differ from the original cells in that their nuclei stain more deeply with basic dyes, and the presence of numerous mitoses indicates that they are dividing rapidly. Mitotic figures are especially numerous in the deeper layers of this newly formed epithelium. As these new cells become more numerous and form layers beneath or in place of the original epithelium, the cells nearer the surface are more and more flattened, the nuclei become larger and contain less chromatin. Later the superficial cells are quite flat, the cytoplasm is homogeneous and the nuclei are lost (Figs. 5 and 6). In this stage the cell boundaries become less clearly defined and at the surface the cells are desquamated as hard, dry, flattened scales. Pasini's waterblue-orcein method shows keratohyalin granules scattered irregularly in the cytoplasm of these cells. In the outer layers these granules sometimes fuse and form hyalin masses of eleidin. The process resembles very closely the incomplete keratinization of the mucous membranes, especially that of the esophagus and certain parts of the tongue of chickens. The columnar ciliated epithelium lining the trachea and bronchi and the epithelium of the submucous glands thus become transformed into squamous stratified keratinizing epithelium with typical intercellular bridges. Owing to the fact that the keratinized epithelium extends as a

continuous layer into the glands of the submucosa, the picture strikingly resembles that of the mucous membrane of the cavity of the mouth, and to a less extent of the normal skin. This is brought out in Fig. 6. Eosinophilic leucocytes which are normally found in the keratinized epithelium of the tongue and esophagus appear either scattered or in small foci. Other cells which may be associated with secondary bacterial infections also appear.

Along with the formation of this keratinized epithelium the original columnar cells are thrown off either singly or in layers. Some of these cells may appear practically normal, but the majority lose their cilia, the protoplasm takes the routine stains less intensely, and the cells later become distended so that they have the appearance of a ball or a balloon (Fig. 7). The chromatin in the nuclei of these cells becomes irregular and fragmented, and later only a few chromatin knots remain scattered irregularly in the balloon-shaped cytoplasm. Finally the cells appear as more or less homogeneous shadows, the whole process being that described by Unna as "balloon degeneration." This balloon degeneration involves not only the original epithelium but also the squamous keratinizing cells. Occasionally these degenerating cells may fuse and form what may be called "balloon giant cells." The cell boundaries may still be visible in these unusually large giant cells.

In the deeper layers of the new epithelium a reticular degeneration of cells may be found. The cells are enlarged, the cytoplasm is occupied by round vacuoles, sometimes to such an extent that it is only visible as a reticular network, the nuclei show numerous chromatin clumps, and nuclear material may be extruded into the cytoplasm. This type of degeneration is not as common as the balloon type.

The degenerating cells become separated and collect in the lumen of the trachea and bronchi in the form of a membrane in which numerous bacterial colonies appear. These bacteria may invade the cells and in cases become so numerous that the cytoplasm is filled with them and the nuclei sometimes destroyed (Fig. 7).

Stains with Sudan III and Scharlach R of the trachea at various stages of the degeneration show practically no fat. In a few cases a small amount of fat was present but this type of degeneration is far from common.

As the keratinization of the epithelial cells of the glands in the trachea progresses, the presence of mucus, as shown by special stain, becomes progressively less. In the early stages one may find keratinized epithelium in one portion of the gland and secreting mucus cells in another. Later, as the keratinized epithelium replaces the true glandular epithelium, mucin is found, if at all, in very small granules in the center of what remains of the original epithelium. When the glands become completely filled with keratinized squamous epithelium mucin is absent.

Associated with the loss of cilia and the decreased or absent secretion of mucin one would expect an invasion of the mucosa with bacteria. Organisms are found not only on the surface of the epithelium and between the epithelial cells but also in the glands of the tunica propria, in the tunica propria itself, and even in the submucosa. The degenerating cells of the epithelium soon become invaded by

bacteria and leucocytes may make their appearance. As the disease progresses bacteria and signs of inflammation become less marked. However, signs of bacterial infection are not always present, and in the fowl we feel that the keratinization of the epithelium is not related to the bacterial infection. In the rat, Tyson and Smith (5) came to the opposite conclusion.

In nearly all cases, a more or less marked cell infiltration in the tunica propria, partly around the numerous vessels, partly in a more diffuse distribution, is found (Figs. 5 and 6). According to the results of control investigations, the occurrence of lymphoid tissue in the tunica propria of the trachea is a normal finding, but in cases of A-avitaminosis its collection is so massive that there is no doubt of its pathological significance. The greater part of these infiltration cells are lymphocytes, to which are added typical histiocytes and a few plasma cells. In most cases some eosinophile leucocytes, red blood corpuscles and occasionally neutrophile leucocytes have also been found. In some early stages with extreme degeneration and proliferation of the original epithelium and a distinct edematous condition of the underlying propria, the occurrence of peculiar large cells, a type of histogenous mast cells, has been observed, especially in the upper layers. They are elongated or oval with a longish-oval nucleus which is rich in chromatin and sometimes shows a typical wheel form. Using Giemsa's stain, very small blue or blue-violet granules can be found in their cytoplasm. In general one has the impression that the upper layers of the tunica propria, situated directly beneath the epithelium of the surface, and the neighborhood of the glands are occupied more densely with these infiltrating cells than its deeper parts. Sometimes the reverse is true; in fact, the degree of the infiltration varies greatly in different cases. Mitoses are very seldom recognizable in these cells. Moreover, lesions may also be found in the endothelial cells of the infiltrated vessels, the lumina of which are frequently filled with lymphocytes. There is more or less swelling of these cells and a change in the chromatin structure of their nuclei. In some preparations the elastic fibers seem to be thickened and more separated. Usually these lesions are associated with edema. In the remaining parts of the trachea no particular changes could be discovered.

The same complex of lesions described in the trachea is also present in the bronchi and bronchioli. They are sometimes filled with desquamated keratinized epithelium, so that bronchiectasis is the natural sequence.

Nasal Cavities and Communicating Sinuses.—The lesions of the nasal passages and their sinuses have been studied on cross sections cut perpendicular to the roof of the mouth at several points between the nostrils and the posterior limit of the nasal cavity or bulb of the eye. The evolution, course, and final stage of the process are exactly the same as previously described in the trachea (Figs. 8, 9, and 10). It may be pointed out that in general all parts of the nasal cavities are

involved and that only some chronological differences in the involvement of the several regions can be observed. In the gland-free part of the nasal vestibule, regio vestibularis, lesions have been determined in all cases, not so much in the form of a real keratinization but rather of an increased proliferation of the superficial epithelial cells.

Typical and distinct lesions, as described in the trachea, are found in the respiratory mucous membrane of the roof of the nasal vestibule and especially in the median and dorsal area of the part known as the concha of the vestibule. One obtains the impression that the entire process in this and other parts of the nasal cavities (olfactory portion, respiratory portion) (Figs. 8, 9, and 10) begins in the mucous membrane epithelium and that the epithelium of the glands becomes involved a little later.

In addition to numerous keratohyalin corpuscles in the cells of the superficial layers of the newly formed squamous stratified epithelium and the appearance of eleidin (Pasini's method), the following lesions in the nuclei of the keratinizing cells have been encountered in the nasal cavities:—As a rule it has been observed, even in the deeper layers, that marked alterations of the nuclei are coexistent with the formation and appearance of keratohyalin granules in the cytoplasm. There is especially a marked fragmentation and extrusion of nuclear material (nucleoli) and furthermore a relative increase in the volume of the cytoplasm as compared with nucleus (Fig. 11). According to the investigations of Ludford (8), the same lesions may take place in the skin under normal conditions, while in a hypertrophied epidermis, under pathological conditions, these nucleolar changes are still more marked. It seems very probable, therefore, that the nuclear substances and particularly the nucleoli are connected in some way with the process of keratinization and the formation of keratohyalin granules. In addition the nucleoli after extrusion from the nuclei not infrequently change their staining properties. When stained by Hammerschmidt's method they resemble Guarneri's inclusion bodies. Moreover, the nuclei of these cells from which the nucleoli have been extruded have the characteristic shape of nuclei in cells containing Guarneri's bodies. It is worthy of note that in the pathological keratinization associated with chicken-pox Eberbeck (9) found the same type of lesion.

The sinuses communicating with the nasal cavity show essentially the same lesions as in the epithelium of the nose and trachea. These lesions are modified depending upon the anatomical structure of the cavity itself. In the paranasal sinuses the mucous membrane is thinner and contains only a few glands on the median wall, contrary to the opinion of Bittner (10). Keratinization occurs in the nasolacrimal duct and in the excretory duct of the lateral nasal gland, *glandula lateralis nasi*. The lateral nasal gland itself is only slightly involved.

the main lesion being in the ducts and collecting space while the acini themselves appear normal.

Bacterial invasion of the degenerating epithelium occurs in the nose just as it does in the trachea (Fig. 12). It is, however, more marked and the nasal cavity and fossa may become filled with degenerated cells and purulent exudate. The tunica propria becomes involved and in addition to the infiltrating cells one may find a slight edema and a swelling of the endothelial cells of the blood vessels.

Relation of A-Avitaminosis to Some Infectious Diseases of the Upper Respiratory Tract

The great variability of the clinical symptoms and pathological lesions in A-avitaminosis of the respiratory tract may make it difficult to differentiate this condition from coryza contagiosa, virus-diphtheria, and infectious tracheo-bronchitis. In the presence of a simple nasal discharge and a discharge from the cleft palate, with involvement of the sinuses, and in the absence of typical pustule-like lesions in the mouth and esophagus, there is a close resemblance to coryza contagiosa as well as to a certain stage of virus-diphtheria (Riedmüller (11)). Recent observations have shown that in such cases, from the clinical, gross anatomical, and even etiological aspect, a differentiation is quite impossible. Furthermore in later stages, after the sinuses and the eyes have become involved and when lesions are present in the cavity of the mouth (12), the A-avitaminosis frequently is almost indistinguishable from the commonly occurring roup and virus-diphtheria. The white color of the caseous masses associated with A-avitaminosis is usually considered to be valuable for the differentiation from the more yellowish products in roup and virus-diphtheria; yet these differences, according to our experience, are by no means distinct enough. Finally, in the absence of typical lesions in other organs, difficulties may arise in distinguishing the disease from infectious tracheo-bronchitis which in certain stages shows a very similar picture in the larynx, trachea, and bronchi.

It will be the object of further investigations to discover possible relations between A-avitaminosis and the development of these infectious diseases of the upper respiratory tract. In this problem, as well as for the purposes of differential diagnosis, the most important matter

is a positive recognition of a present lack of vitamin A. The histological lesions which occur in the respiratory tract and throughout the body even in early conditions, taken in connection with the more recent investigations of Wolbach and Howe (5), Goldblatt and Benischek (3), and Tyson and Smith (4), who were able to produce similar lesions in rats and guinea pigs by feeding a diet lacking vitamin A, seem definitely to prove the specificity of these lesions. It may be added that in cases of so-called "coryza contagiosa" without a lack of vitamin A quite different lesions have been found.

Only by means of the histological examination is it possible to differentiate the A-avitaminosis from the above mentioned infectious diseases of the respiratory tract.

SUMMARY AND CONCLUSIONS

1. The principal tissue changes in the respiratory tract of chickens caused by a vitamin A deficiency in the food are, first, an atrophy and degeneration of the lining mucous membrane epithelium as well as of the epithelium of the mucous membrane glands. This process is followed or accompanied by a replacement or substitution of the degenerating original epithelium of these parts by a squamous stratified keratinizing epithelium. This newly formed epithelium develops from the primitive columnar epithelium and divides and grows very rapidly. The process appears to be one of substitution rather than a metaplasia, and resembles the normal keratinization of the skin or even more closely the incomplete keratinization of the mucous membranes (e.g., the esophagus or certain parts of the tongue of chickens). In this connection findings have been described which not only afford an interesting insight into the complicated mechanism of keratinization, but also show probable relations between keratinization and the development of Guarnieri's inclusion bodies. Balloon and reticular degeneration of the upper layers of the new stratified epithelium has been frequently observed. All parts of the respiratory tract are about equally involved in the process; and the olfactory region as well, so that the sense of smell may be lost. The lesions, which first take place on the surface epithelium and then in the glands, show only minor differences.

2. The protective mechanism inherent in the mucous membranes of

the entire respiratory tract is seriously damaged or even entirely destroyed by the degeneration of the ciliated cells at the surface and the lack of secretion with bactericidal properties. Secondary infections are frequently found, and nasal discharge and various kinds of inflammatory processes are common, including purulent ones, especially in the upper respiratory tract, communicating sinuses, eyes and trachea. The development of the characteristic histological process is not dependent upon the presence of these infections, since it also takes place in the absence of infection.

3. The specific histological lesions make it possible to differentiate between A-avitaminosis and some infectious diseases of the respiratory tract.

These studies we hope will serve as a basis for further investigations on the relationship between A-avitaminosis and infection in general.

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EXPLANATION OF PLATES

PLATE 21

FIG. 1. Sagittal section through the head of Chicken 7 with A-avitaminosis. Masses of purulent exudate in the nasal passages. (Dead after 82 days on experimental diet.)

FIG. 2. Cross section through the head of Chicken 13 with A-avitaminosis (spontaneous case). White caseous masses between left concha inferior and nasal septum and in the corresponding sinuses.

FIG. 3. Trachea of Chicken 6 with A-avitaminosis. Thin membranes, partly in the form of a tube, consisting of desquamated epithelium. (Dead after 87 days on experimental diet.)

FIG. 4. Trachea, cross section (84.5 \times). Chicken 1. Beginning stage. Degeneration of the surface epithelium and separation from its underlying support. On the left side formation of a new stratified epithelium in a small focus. (Killed after 30 days on experimental diet.)

FIG. 5. Trachea, cross section (50 \times). Chicken 8. Complete replacement of the epithelium of the respiratory mucosa and its glands by a stratified keratinizing epithelium. Infiltration of the submucosa. (Dead after 80 days on experimental diet.)

PLATE 22

FIG. 6. Same as Fig. 5 (80 \times).

FIG. 7. Cross section through trachea (990 \times). Chicken 1. Newly formed stratified epithelium. At the surface several cells showing balloon degeneration. Foci of bacteria between the degenerating cells. (Dead after 30 days on experimental diet.)

FIG. 8. Nasal septum (50 \times). Chicken 6. Complete replacement of the surface epithelium by stratified keratinized epithelium. Desquamation of the superficial layers. The glands show an earlier stage of the process, atrophy of the original epithelium. Slight infiltration of the submucosa. (Dead after 87 days on experimental diet.)

FIG. 9. Olfactory region (85 \times). Chicken 6. Advanced keratinization and desquamation of the replacing epithelium at the surface of the mucous membrane. The epithelium of Bowman's glands shows only the typical atrophy. Masses of exudate and desquamated epithelium. (Dead after 87 days on experimental diet.)

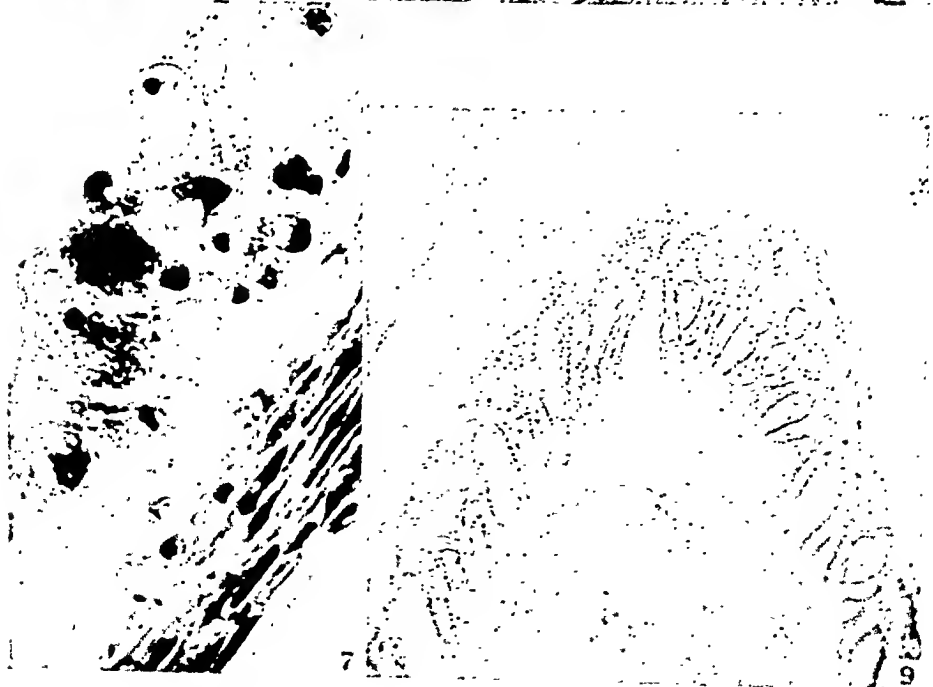
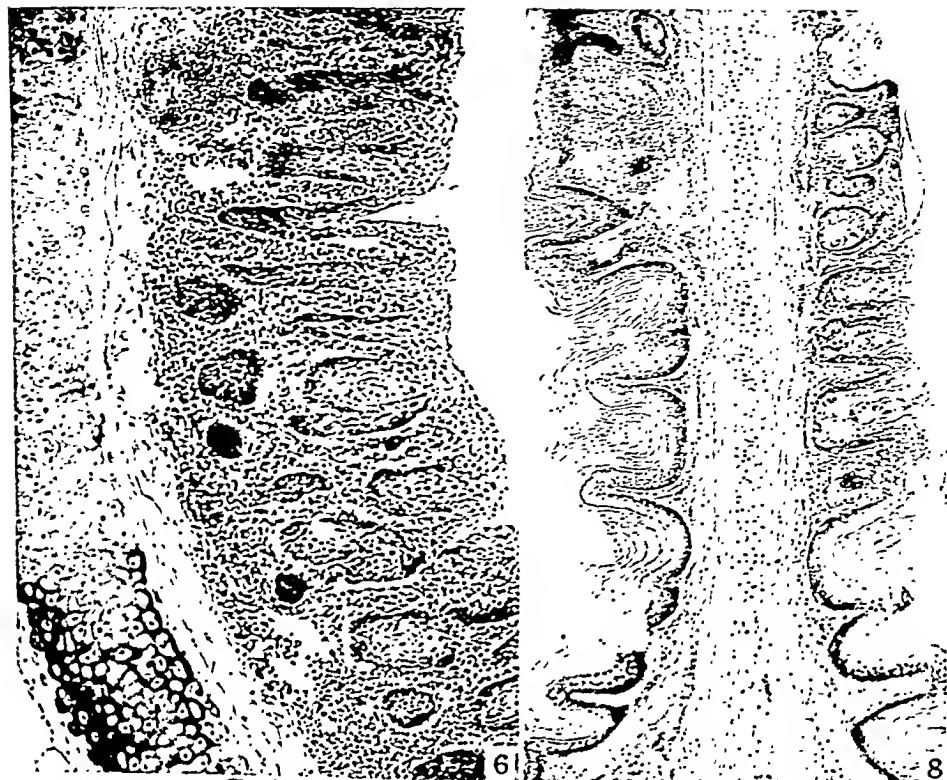
PLATE 23

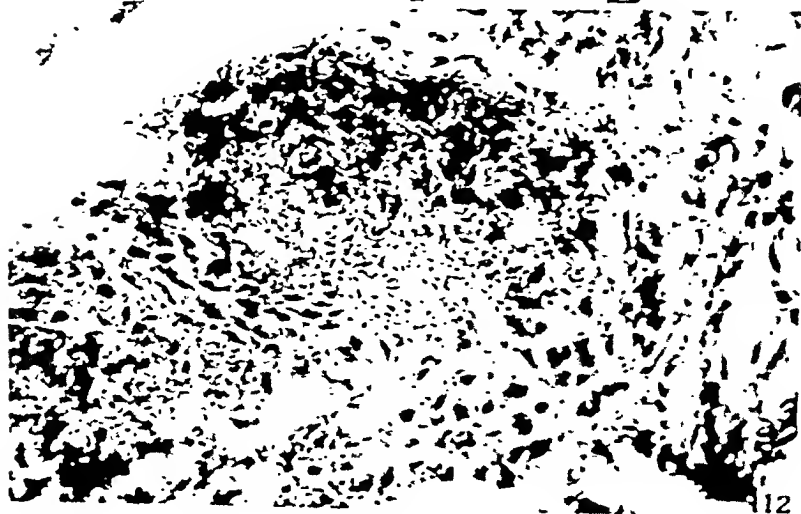
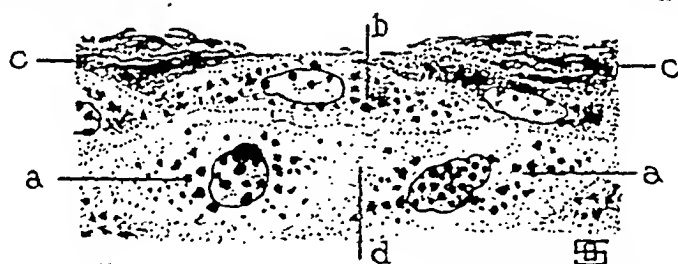
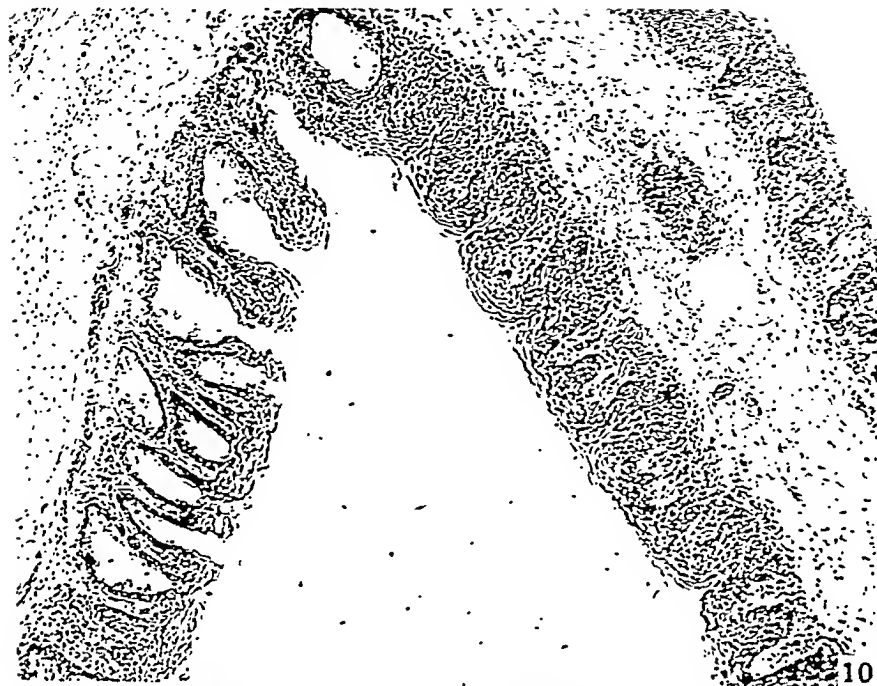
FIG. 10. Respiratory and olfactory mucous membrane of the nasal fossa (55 \times). Chicken 5. Complete replacement by stratified keratinizing epithelium. The glands show an earlier stage of the process. (Dead after 85 days on experimental diet.)

FIG. 11. Gland of the nasal mucous membrane (1140 \times). Chicken 5. Showing numerous bacteria in an early stage of the process. (Dead after 85 days on experimental diet.)

FIG. 12. Nasal passages. Chicken 6. Cells of the superficial layers of the newly formed keratinizing epithelium, showing the extrusion of nuclear material into the cytoplasm (a), granules of keratohyalin (b), masses of eleidin (c), and intercellular bridges (d).







STUDIES ON A-AVITAMINOSIS IN CHICKENS

II. LESIONS OF THE UPPER ALIMENTARY TRACT AND THEIR RELATION TO SOME INFECTIOUS DISEASES

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PLATES 24 AND 25

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In the previous paper¹ of this series the lesions in the respiratory tract caused by a lack of vitamin A in the food of chickens was considered, together with a discussion of the clinical findings and their relation to certain infectious diseases. Using the same material and methods, it is the purpose now to deal in the same way with the lesions of the mouth, palate, esophagus, crop, and their associated glands.

Gross Pathology

Because of the close connection between the nasal cavity and the mouth, the seromucous discharge in the former overflows regularly through the cleft palate and produces a clinical and anatomical picture which cannot be differentiated from that found in coryza contagiosa. As secondary invaders appear this exudate becomes transformed into white or slightly yellowish caseous masses which may at times completely plug the cleft palate. In addition, in the region of these nasal plugs and also on the roof of the mouth but seldom in the esophagus, fine thin membranes appear which together with the nasal plug produce a picture which may be easily confused with that of fowl-pox. In A-avitaminosis, however, these membranes may not always be present and when they are they usually are limited to the cleft palate and its adjacent epithelium. They are easily removed leaving no bleeding ulceration, thus differing from fowl-pox.

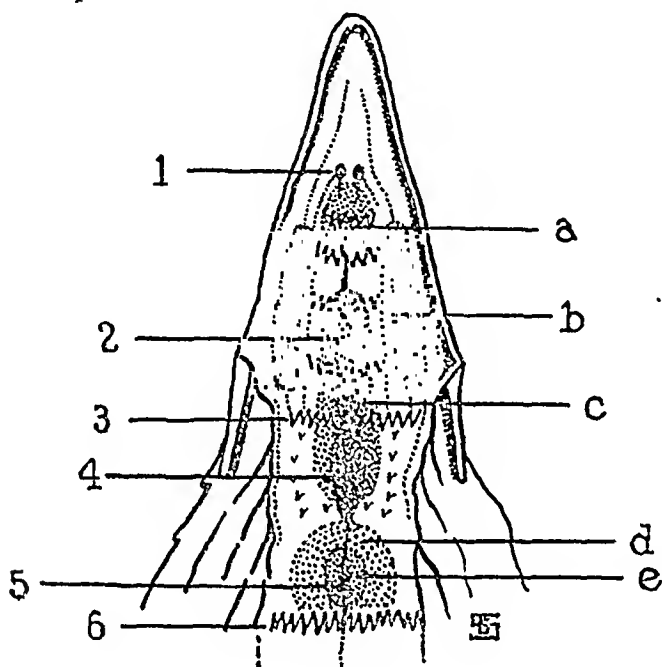
Early in A-avitaminosis there appear small white or yellowish pustule-like lesions, varying from 0.5 to 2 mm. in diameter, in the region of the excretory ducts of the glands of the mouth (Text-figs. 1 and 2). When discrete they are round, but by confluence they may become longish. They are usually raised above the

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¹ Seifried, O., *J. Exp. Med.*, 1930, 52, 519.

surface of the mucous membrane and often show a depression in the center (Fig. 1). Later small ulcers may appear at the site of these lesions and these ulcers may be surrounded by inflammatory products. These pustule-like lesions, with or without the inflammatory membranes, very closely resemble lesions of fowl-pox, and it is with great difficulty, if at all, that a differential diagnosis can be made by the macroscopical picture.

The mucosa of the esophagus and crop also shows pustule-like patches which may be even more prominent than those seen in the mouth, probably due to the



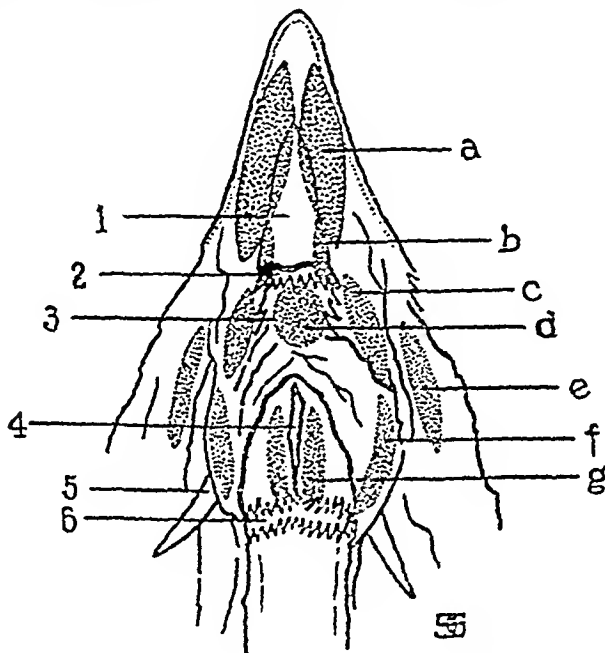
TEXT-FIG. 1. Roof of mouth. 1: excretory ducts of maxillary gland; 2: cleft palate, narrow part; 3: papillae of palate; 4: cleft palate, wide part; 5: infundibulum; 6: papillae of throat. a: glandula maxillaris; b: glandula palatina lateralis; c: glandula palatina medialis; d: glandula spenopterygoida; e: glandula tubaria.

peculiar structure of the glands in these regions (Fig. 1). They may be either localized in the upper part of the esophagus and surrounding the pharynx, or they may cover the mucous membrane of the esophagus throughout its entire length and in cases extend into the posterior part of the crop where the folds of the esophagus are continued. In the main portion of the proventriculus and throughout the intestines no gross lesions have been found. The nodules in the esophagus do not ulcerate as frequently as those in the mouth cavity, but they show a depressed minute opening in the center which corresponds to the excretory duct of the gland. In a few instances they are so numerous in the esophagus that when infected a type of membrane may be formed.

Histology

As noted above the lesions in the alimentary tract occur mainly in the glands. They have been studied in sections made of the roof of the mouth after decalcification.

Glands from the floor of the mouth, and in some instances from the roof, have been dissected out and sectioned in their shortest and longest diameters, while the



TEXT-FIG. 2. Floor of mouth. 1: tongue; 2: papillae of tongue; 3: base of tongue; 4: entrance to larynx; 5: bone of tongue; 6: papillae of larynx. a. glandula submaxillaris anterior; b: glandula lingualis anterior; c: glandula submaxillaris oromedialis; d: glandula lingualis posterior; e: glandula submaxillaris caudolateralis; f: glandula submaxillaris intermedia; g: glandula cricoarytenoidea.

glands of the tongue, pharynx and esophagus have been sectioned *in situ*. The lesions in the glands are essentially the same as those found in the respiratory tract and described in the previous paper. There are, however, some peculiarities which probably depend upon the special structure of the glands. As a rule the early lesions in these glands are not found in the acini but in the collecting spaces and ducts. Lesions may be well marked in the maxillary and submaxillary glands before they appear in the nasal passages, sinuses, trachea or bronchi. Fig. 2 shows

an early lesion of the submaxillary gland in which there is a marked proliferation and degeneration of the original epithelium. At the same time new stratified epithelium appears beneath or in place of the original epithelium. These early lesions may not be very characteristic but as the process is advanced typical pictures occur, as shown in Fig. 3 of the maxillary gland where in the collecting space is found a focus of squamous stratified keratinizing epithelium. In the acini the high mucus-secreting epithelium has begun to degenerate and atrophy and in a few instances there has been a replacement by stratified keratinizing cells. These changes in the acini have never been as pronounced as in the collecting spaces. Fig. 4, a cross section through the *glandula lingualis*, shows a well marked stage in the process with newly formed stratified epithelium and early keratinization. This newly formed epithelium may be found in isolated islands or in larger foci beneath or in place of the original epithelium lying directly upon the connective tissue. It seems probable that this newly formed tissue is favored in its growth by the abundant supply of capillaries from the surrounding connective tissue. The collecting spaces become filled with degenerated cells, masses of mucus (Fig. 4), and in some cases inflammatory products. This accumulation is undoubtedly in large part due to the lesions in the excretory ducts of the glands, as it is here that the oldest and most pronounced changes have been found. The epithelium of the mucous membrane of the mouth seems to extend into the duct, which is partially filled with the new stratified epithelium, and causes a more or less complete blocking. As a result the glands become dilated and frequently show evidences of bacterial infection, the latter being especially marked in the duct. Since this is an early lesion the gland may continue to secrete mucus for a time and this accumulates in the collecting space. Later desquamated cells from the newly formed stratified epithelium become numerous and the gland, which originally is a sac with invaginations, becomes smoothed out and distended. Finally this distended sac becomes completely filled with stratified keratinized epithelial cells, as shown in Fig. 5 which gives a picture of the final stage of the process. Such a picture is found more frequently in the glands of the tongue, palate and esophagus than in the compound glands of the cavity of the mouth.

The histological picture shows that early infections of the excretory ducts of the glands are relatively common. These infections are manifested by necrosis of the epithelial lining of the duct and of the surrounding mucous membrane, cellular infiltrations, and the presence of numerous bacteria (Figs. 6 and 7). Oftentimes it appears that the lumen of the duct is blocked, thus resulting in an accumulation of inflammatory products in the gland itself. At times the inflammatory process spreads from the mouth of the duct over the surrounding mucous membrane of the mouth (Fig. 8) or tongue, and more rarely over the mucous membrane of the esophagus.

In the submucosa signs of infection such as perivascular and diffuse infiltrations are frequently seen. In some sections through the tongue such infiltrations have been found even in the deeper layers of the muscularis, and in the ventral

portion of the vomer these lesions are particularly pronounced. In the esophagus and crop and sometimes in the proventriculus, the lesions in the glands are the same as those described in the mouth cavity but secondary bacterial infections are not as pronounced.

As pointed out above, the macroscopical lesions in the mouth may closely resemble lesions of fowl-pox. The histological difference between these two is quite striking and there is no difficulty in separating them from sections, but what part the process plays in the development of fowl-pox and other infections by breaking down the resistance of the mucous membranes is a problem that is well worth further consideration.

SUMMARY AND CONCLUSIONS

When fowls are placed on a diet lacking in vitamin A lesions appear in the upper alimentary tract which are confined largely to the mucous glands and their ducts. Histologically it is shown that the original epithelium becomes replaced by a stratified squamous keratinizing epithelium and that secondary infections are relatively common. The ducts of the glands may be blocked leading to distention with secretions and necrotic materials. These lesions macroscopically resemble very closely certain stages of fowl-pox and the two conditions can be separated only by histological examination. It is pointed out that these lesions produced by a lack of vitamin A may enable bacteria and other viruses to enter the body.

EXPLANATION OF PLATES

PLATE 24

FIG. 1. Pustule-like patches at the roof of the mouth, the base of the tongue and the mucous membrane of the esophagus. (Chicken 7, dead after 82 days on experimental diet.)

FIG. 2. Longitudinal section through the glandula submaxillaris of Chicken 11. $\times 55$. Proliferation of the original epithelium lining the collecting space; first stage of process. Hematoxylin-eosin. (Killed after 56 days on experimental diet.)

FIG. 3. Cross section through the maxillary gland of Chicken 1. $\times 110$. Small foci of stratified keratinizing epithelium in the collecting space; degeneration of the epithelium in the acini. Hematoxylin-eosin. (Killed after 39 days on experimental diet.)

FIG. 4. Cross section through the base of the tongue (glandula lingualis) of Chicken 9. $\times 80$. Shows a more advanced stage of the process. The cells, which are to replace the original epithelium, form a continuous vascularized syncytium, lying directly upon the surrounding connective tissue. Beginning keratinization, desquamation and degeneration of the upper layers of the newly formed epithelium. Hematoxylin-eosin. (Dead after 75 days on experimental diet.)

PLATE 25

FIG. 5. Cross section through the base of tongue (glandula lingualis) of Chicken 6. $\times 50$. Final stage of the entire process. The glands are completely filled with stratified, more or less keratinized, homogeneous masses. Extreme dilatation of the glands. van Gieson stain. (Dead after 87 days on experimental diet).

FIG. 6. Excretory duct of the glandula palatina medialis of Chicken 5. $\times 50$. Bacterial infection followed by necrosis of the duct and its surroundings; accumulation of necrotic masses, mucus, inflammatory products and numerous bacteria. Giemsa stain. (Dead after 85 days on experimental diet.)

FIG. 7. Excretory duct of the lateral glandula palatina of Chicken 7. $\times 875$. Invasion with numerous bacteria. Methylene blue stain. (Dead after 82 days on experimental diet.)

FIG. 8. Cross section through roof of mouth (glandula maxillaris) of Chicken 7. $\times 75$. Thick membrane covering the mucous membrane, developing after secondary infection of the excretory ducts. Hematoxylin-eosin. (Dead after 82 days on experimental diet.)





THE DISTRIBUTION OF FRIEDLÄNDER'S BACILLI OF DIFFERENT TYPES

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In 1926 (1), a number of strains of Friedländer's bacillus were classified by the immunological reactions of agglutination, agglutinin adsorption and passive protection against infection, into three specific types, which were arbitrarily designated as Types A, B, and C. The strains falling into the specific types comprised the great majority of those studied, and the remaining cultures which were found to differ serologically from each other and from the specific types were included in a heterogeneous group called Group X. The distinctions in type were referable to the presence of a specific carbohydrate in the capsular material. The carbohydrate (2), or soluble specific substance, was shown to be chemically different for each of the three types.

Cultures of Friedländer's bacillus have been collected in the meantime in order to study the relative frequency and distribution of the organism of various immunological types.

EXPERIMENTAL

In the present survey a total of 80 strains have been isolated from a variety of disease conditions; in most instances the organisms were considered to be the inciting agent of the disease, in other cases secondary invaders. All the organisms studied were encapsulated, non-motile, non-spore-bearing, Gram negative rods with the cultural characteristics usually attributed to Friedländer's bacillus. In each instance the serological type was determined by the agglutination of live young cultures (4 to 6 hours) in type-specific sera.

The fermentation of carbohydrates by the different strains was also studied to determine possible serological and biochemical relationships.

The Frequency of Occurrence of Friedländer's Bacilli of the Different Specific Types.—In Table I is given the relative number of strains of the different types isolated from disease. It is seen that of the 80 strains, about 75 per cent fall in one or other of the three specific types. To Type A, belong 42 of the strains (52 per cent); to Type B, 12 (15 per cent); to Type C, 7 (9 per cent). Group X includes 19 (24 per cent) of the cultures. It is interesting to compare these figures with those obtained from the original study (1) of 30 strains. At that time, it was found that 50 per cent of the strains were of Type A, 20 per cent of Type B, 10 per cent of Type C, and 20 per cent belonged in Group X.

The Incidence of the Various Specific Types in Different Diseases.—This is shown in Table II. Most of the strains studied were isolated from cases of pneumonia in man. Of the strains isolated from this

TABLE I
The Distribution of Specific Types of Friedländer's Bacillus

Total number of strains studied	Distribution by types			Group X
	Type A	Type B	Type C	
80	42	12	7	19

source about 75 per cent were of Type A. In the original study (1) it was found that about 70 per cent of the total strains associated with pneumonia belonged to Type A. Most of the Type A strains were isolated from human cases where the majority of Type B strains were isolated from animals. The organisms of Group X were obtained from a variety of conditions in animals and man.

The Specific Precipitin Reaction in the Urine in Pneumonia.—In a preceding paper (3) it was shown that rabbits experimentally infected with Friedländer's bacillus eliminate the soluble specific substance through the kidney, and that the urine of infected animals may give a precipitation reaction in the corresponding type-specific serum. Blake (4) first demonstrated the presence of a positive precipitin reaction in a case of pneumonia in man due to Friedländer's bacilli. Recently in a case of pneumonia in the Hospital of The Rockefeller Institute, due to Friedländer's bacillus, the opportunity was offered

for studying the type-specific precipitin reaction in the urine. On the day of admission, which was the second day of the disease, Friedländer's bacillus Type A was isolated both from the blood and sputum, and the presence of the soluble specific substance was demonstrated in the urine by a type-specific precipitation reaction which was positive even when the urine was diluted sixteen times. On the following

TABLE II

The Incidence of the Various Types of Friedländer's Bacillus in Different Diseases

Type	Human pneumonia	Throat	Liver abscess	Animal source	Miscellaneous
Type A (42 strains)	33	3	1	Guinea pig—2 (not pneumonia) (fatal abscess)	Cystitis—1 Adenoid tissue—2
Type B (12 strains)	2	1	1	Guinea-pig—5 (pneumonia) Mare—2 (G. U. infections)	Feces—1 (colitis)
Type C (7 strains)	3	0	0		Antrum—1 (sinusitis) Nose—1 (sinusitis) Unknown—2
Group X (19 strains)	7	2	1	Guinea-pigs—2 (not pneumonia) (fatal abscess)	Sputum—2 (not pneumonia) Cystitis—3 Lung abscess—1 Feces—1 (Pellagra)

day the disease terminated fatally and this day the precipitation reaction was positive when the urine was diluted 1:64.

The Fermentation of Carbohydrates by Friedländer's Bacilli of the Different Types.—The fermentation reactions of bacteria of the *Encapsulatus* group have been studied by a number of workers, but the results have been contradictory and confusing. While certain authors have found that Friedländer's bacillus ferments lactose with the formation of acid and gas (5, 6, 7, 8) or of acid alone (9, 10, 11, 12), other writers (13, 14, 15) have observed that it does not ferment

lactose. Still other authors (16, 17, 18, 19) state that different strains may vary in their ability to ferment lactose, and it is the opinion of these authors that the fermentation reactions of the bacteria of the

TABLE III

The Fermentation Reactions of Friedländer's Bacillus of the Different Specific Types

Friedländer bacilli		Reactions in				
Type	Number of strains	Dextrose	Lactose	Sucrose	Maltose	Mannitol
A	30	AG	AG	AG	AG	AG
B	8					
C	3					
Group X	8					
A	9	AG	Negative	AG	AG	AG
B	None					
C	1					
Group X	1					
A	2	A	A	A	A	A
B	2					
C	None					
X	5					
A	4	A	Negative	A	A	A
B	None					
C	None					
X	None					
X	1	AG	AG	Negative	AG	Negative
X	1	A	Negative	Negative	Negative	Negative
X	1	A	Negative	Negative	AG	Negative

A indicates presence of acid.

AG indicates presence of acid and gas.

Negative indicates absence of acid and gas.

Encapsulatus group are too inconstant to be of value in classification or identification.

It seemed, however, important to study again this question with reference to the specific types of organism employed in making the tests.

Five carbohydrates (dextrose, lactose, sucrose, maltose and manitol) were employed, since earlier observations indicated that these five offered the greatest possibilities for use in differentiation. Samples of meat extract broth containing 1 per cent of the respective carbohydrates were inoculated, and the final readings were made after an incubation period of 7 days.

The results of this study are given in Table III. It is seen that the majority of the strains ferment all 5 carbohydrates with the formation of acid and gas. There is, however, a great variability in the fermentative activities of the different strains, and it is obvious that a classification of Friedländer's bacilli on the basis of fermentation activity is of little value. Furthermore, the fermentation reactions do not aid in the identification of the specific types. It is interesting to note, nevertheless, that of 15 strains which form neither acid nor gas in lactose, 13 are of Type A. In other words, the inability to ferment lactose is most characteristic of Type A cultures. Of further interest is the fact that the strains of Group X show the greatest variability in the fermentation of carbohydrates.

DISCUSSION

The present communication records the distribution of Friedländer's bacilli of the different specific types in disease. It was found that most of the Type A strains are obtained from human cases and the majority of them from cases of pneumonia. On the other hand, the greatest number of strains of Type B are derived from animal sources, while Group X contains organisms isolated from the greatest variety of diseases. In this connection, it should be pointed out that in confirming the existence of specific types among the Friedländer bacilli, Edwards (18, 19) characterizes his Type I (corresponding to Type B) as the animal type, and Type II (corresponding to Type A) as the human type. Edwards (19) also shows that certain strains of *Bacterium aerogenes* are serologically identical with Friedländer's bacilli. This is not surprising, however, when it is recalled that Avery, Heidelberger and Goebel (20) had previously demonstrated the immunological similarity of Type B Friedländer's bacillus to the more widely remote species, *Pneumococcus* Type II.

It has been possible to confirm Blake's observations on the occur-

rence of a specific precipitin reaction in the urine during the course of pneumonia due to Friedländer's bacillus. Dochez and Avery (21), also demonstrated during their original studies on the soluble specific substance of *Pneumococcus* that the specific urine precipitation tests in immune sera offered a procedure of diagnostic as well as prognostic importance.

A study of the fermentation reactions of Friedländer's bacillus confirms an earlier opinion (17) that a classification based on these reactions possesses little value. Fitzgerald (16) previously and Edwards (18, 19) more recently reached similar conclusions. In the present study, it was not possible to show any constant relationship between serological reactions and biochemical activity as measured by the fermentation of carbohydrates, although it was found that among the strains of all types there is a much greater tendency for Type A strains not to form acid or gas from lactose.

SUMMARY AND CONCLUSIONS

In a study of the distribution of the specific types of Friedländer's bacillus, it is shown that: (1) Of 80 strains 52 per cent belong to Type A, 15 per cent to Type B, 9 per cent to Type C, and 24 per cent to Group X. (2) Type A contains for the most part strains derived from diseases of man and more than 70 per cent are associated with pneumonia in man. (3) Type B includes the greatest number of strains from animal sources, while the heterogeneous strains comprising Group X come from the greatest variety of diseases.

It was demonstrated that in a patient suffering with pneumonia due to Friedländer's bacillus (Type A), a specific precipitin reaction of the urine occurred in the corresponding (Type A) immune serum.

A study of the sugar fermentation reactions of Friedländer's bacillus shows that (1) there is no correlation between serological type and fermentative activity; (2) the fermentation reactions are variable and therefore not reliable for distinguishing Friedländer's bacillus from closely allied organisms; (3) the strains of Group X show the greatest variation in fermentation, and (4) of 15 strains unable to ferment lactose, 13 belong to Type A.

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STUDIES OF DISEASES OF THE LYMPHOID AND MYELOID TISSUES. I.

THE CHEMICAL METABOLISM OF NORMAL AND PATHOLOGICAL LYMPH NODES*

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Warburg and his associates, Posener and Negelein (1924), have shown that within certain limits and with certain exceptions carcinomatous tissue has a unique and characteristic metabolism. They and Warburg (1925) found that normal tissues have a relatively high rate of oxygen consumption and a low aerobic glycolysis. Embryonic tissue has high respiration and, under anaerobic conditions, a high glycolysis, but it has only a slight destruction of sugar under aerobic conditions. Neoplastic tissue, on the other hand, has a rather low respiration rate and a high glycolysis under both aerobic and anaerobic conditions. These broad generalizations have been corroborated by Murphy and Hawkins (1925), Rona and Deutsch (1926), and others. The subject is more fully reviewed by Jackson (1929). Neither glycolysis nor oxygen consumption, however, proved to be an infallible guide to the nature of a given living tissue, so that Warburg (1927) resorted to a new value by which to differentiate neoplastic from normal tissue. He points out that when the Pasteur reaction functions at its maximum, the formation of exactly two molecules of lactic acid is suppressed when one molecule of oxygen is consumed, and on this basis he suggests a new value "U," which may be regarded as the

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theoretical aerobic glycolytic rate. This value U he finds rather constantly negative for normal tissue, in the vicinity of zero for embryonic tissue, and strongly positive for carcinomata.

In man the lymph nodes are subject to various pathological processes including what, for lack of a better term, we may call the malignant lymphomata, a classification which is used in this paper to include lymphosarcoma, Hodgkin's disease, reticulum cell sarcoma and lymphatic leukemia. Pathologists are not entirely agreed as to the nature of these rather varied, but at the same time similar, pathological processes. Some regard them as neoplasms, others as infectious granulomata. Other pathologists again would class some types in one group, some in the other. In this paper we are placing Hodgkin's disease, as defined below, by itself for comparison with the other types of lymphomata.

As a part of a general investigation of this group of diseases it occurred to us that a study of the chemical metabolism of pathological lymph nodes might throw some light on their fundamental nature. The metabolic processes might, we hoped, afford evidence as to whether various types of malignant lymphomata were neoplastic or infectious in origin. This paper deals with the results of the investigation of normal and pathological lymph nodes and primary tumors not involving lymph nodes, from 71 patients, and is the first of a series of papers dealing with various aspects of malignant lymphomata.

Methods

As soon as received from surgical operation, the tissue was cut into thin sections, averaging 0.3 mm. in thickness, by a specially devised knife consisting of two safety razor blades held parallel, but slightly apart, in a suitable metal holder provided with adjustments for aligning the blades. All such sections were immediately immersed in sterile, normal horse serum, at room temperature, preparatory to their transfer to the metabolism chambers. Sterile cultures of the lymph nodes in this horse serum lived as long and as well as in the patient's own serum. Paraffine sections were made of all lymph nodes examined.

The metabolism was carried out in all instances in a modified Thünburg respiration apparatus. Briefly this consisted of two small vials connected one with another by means of a long, very fine capillary tube containing a kerosene drop to serve as a direct indicator of the oxygen consumption. The tissue to be metabolized was floated in buffered normal horse serum in an inner chamber. The moat about this inner chamber contained 2 per cent NaOH which served to absorb all

carbon dioxide formed. The anaerobic glycolysis was determined in a small vial containing horse serum saturated with pure nitrogen which had been passed over red hot copper to free it from traces of oxygen. The serum used in the anaerobic experiments was first subjected to a vacuum to rid it of air and subsequently saturated with pure nitrogen. This anaerobic chamber was attached to the stand supporting the Thünburg apparatus. The whole apparatus, aerobic and anaerobic alike, was immersed in a constant temperature water bath at 37.5°C. and kept agitated by a suitable motor. All apparatus coming in contact with the tissue was chemically clean. That the possible presence of bacteria did not affect the results was evident from several specially devised experiments and also from the constancy, from period to period, of the tissue metabolism, as evidenced by the oxygen consumption. Following each experiment the tissue was washed free of serum, dried at 100°C., and weighed. From 5 to 25 mg. tissue was used. All weights refer to dry tissue.

Oxygen consumption was read directly on a calibrated scale attached to the capillary. Readings were taken every 5 minutes. The results are expressed in c. mm. per mg. hour. Glycolysis, or more accurately, sugar destruction during the experimental period was determined by the familiar method of Folin (1929). The figures given in the text are those actually observed, except that in those tumors in which there was considerable connective tissue stroma a suitable allowance was made as this tissue has a metabolism entirely negligible compared to that of the more active cells composing the rest of the tumor. The amount of stroma present was carefully estimated by two independent observers from paraffine sections. The observed metabolic figures were then multiplied by the appropriate factors. In only 14 of the 71 cases was the amount of stroma increased sufficiently to warrant the application of a correctional factor.

Experimental Results

Normal Nodes.—Four normal nodes were examined. The average aerobic glycolysis was 0.020 mg., the average anaerobic glycolysis 0.054 mg., and the average oxygen consumption 5.7 c. mm. In this small series of cases there was a marked uniformity of the results. The average value U was 4.1, a figure entirely consistent with that found by Warburg for normal lymphoid tissue, but slightly higher than that found in most normal tissues elsewhere in the body which, as has already been stated, usually have a negative U . These figures can probably be taken as representative of the normal lymph node, and must be used as a determining value for further correlations.

Lymphoma.—Under this heading we here include lymphosarcoma, lymphatic leukemia, lymphocytoma, lymphoblastoma, and reticulum

cell sarcoma, but not Hodgkin's disease. There were studied metabolically nodes from 14 such cases. In contradistinction to the normal nodes there was found to be a marked variation in the different factors making up the metabolism in these pathological cases. The average aerobic glycolysis was 0.063 mg., over three times that found in the normal series. Three cases had an aerobic glycolysis above 0.083 mg., and all of these cases were rapidly fatal, while in two cases the rate fell below 0.035 mg., and these also were highly malignant from a clinical point of view. No correlation, therefore, could be found between degree of malignancy and the rate of aerobic glycolysis. The nodes from case S-29-662 and those from case S-29-1574 gave almost identical figures for aerobic glycolysis, yet the former case is alive and well a year and a half later, while the latter case died in a few months. The average anaerobic glycolysis was 0.092 mg. One, a case of rapidly advancing lymphosarcoma (S-29-236), showed the extremely high figure of 0.206 mg., while another, a case of lymphosarcoma involving the stomach (S-29-1585), had a low figure of 0.047 mg. Again, no correlation can be traced. The oxygen consumption averaged 5.5 c. mm. per mg. hour with such extreme variations as 0.7 and 12.6, yet there was no apparent relation between the metabolic rates and either the pathological or the clinical findings. The value U averaged 11.8, thus placing these tumors tentatively in the neoplastic class. But again extreme variations occurred, as witness case S-29-100 A, a rapidly advancing and rapidly fatal lymphoma with metastases to bone which had a U value of -1.7 , and, on the other hand, S-29-236, a lymphosarcoma with a U of $+47$ and fatal in approximately the same time as the first case. Again case S-29-662 had a U value well above the general average for this class and well within the limits set by Warburg for malignancy, yet the case responded in a remarkable way to high voltage X-ray therapy and has remained well over a period of nearly 2 years.

Hodgkin's Disease.—Eighteen cases of typical Hodgkin's disease were studied. We class as Hodgkin's disease those nodes which show the typical multi-nucleated giant cells described by Dorothy Reed and by Sternberg. The tumor in some of the cases was very cellular, while in others it was sclerosed. The average aerobic glycolysis was 0.052 mg., slightly lower than the corresponding figure for all other types of lymphoma. There was a remarkable tendency for the figures to vary

about the average within narrow limits, the lowest being 0.030 mg. and the highest 0.067 mg. The average oxygen consumption was 5.8 c. mm. The anaerobic glycolytic rate was 0.074 mg., again slightly lower than that in the other lymphomata. The value U averaged 7.3, slightly above the corresponding value for normal nodes but definitely below that for tuberculous nodes which was 10.3. From this it might be inferred that Hodgkin's disease was infectious in nature, or at least not neoplastic, but it should be remembered that the lymphosarcoma S-29-100A had a value U of only -1.7, and another lymphosarcoma infiltrating the stomach and the parotid had a value of 3.4. On the other hand, a case of sclerosing Hodgkin's disease (S-29-1152), responding well to X-ray therapy, showed in the node a value U of 19.6, while another case, histologically similar and clinically rapidly fatal (S-29-1556) had a low value of 0.9. So it must be recognized that no very clear evidence is obtained as to the fundamental nature of the lesions from these data.

Tuberculosis.—Four cases of the cellular type of tuberculosis were studied. Necrotic or very fibrous nodes were excluded for obvious reasons. Here it was found that the average aerobic glycolysis was 0.055 mg., and the average anaerobic 0.090 mg. The oxygen consumption ran high, averaging 8.2 c. mm., while the U averaged 10.3. Nye and Parker (1930) have produced in animals lesions consisting of closely packed mononuclear cells identical with, or very similar to, those found in cellular tuberculosis. The lesion is particularly prominent and particularly pure in the lung, and examination of the involved lungs from these animals showed that they had an aerobic glycolysis of 0.05 mg. and anaerobic of 0.09 mg., a U of 15. The similarity between these figures and those of human tuberculosis is obvious, and the figures indicate that the type cell rather than the etiological agent or the fundamental nature of the disease is responsible for the character of the metabolism. If one attempts to classify tuberculosis on the basis of Warburg's figures one is forced to admit that here again is an obvious exception to his general rule; for the observed figures alone would indicate rather sharply that the process is neoplastic.

Carcinoma.—Thirteen cases of carcinomata involving lymph nodes were examined. Here again, as in the case of Hodgkin's disease, due allowance was made, when necessary, for the presence of large amounts of physiologically inactive connective tissue. The average aerobic

glycolysis was 0.088 mg., a figure far higher than any other tissue previously examined, but rates as low as 0.032 mg. (S-29-1042) were found in a slowly growing, highly differentiated epidermoid carcinoma. Five cases had an aerobic glycolysis above 0.100 mg., and 10 were above 0.060 mg. The average anaerobic rate was 0.142 mg., but varied considerably. With one exception, case S-29-1042 again, all were above 0.068 mg., and six cases yielded figures which were above 0.150 mg. Oxygen consumption was consistently low, averaging 4.1 c. mm. As was to be expected from Warburg's work, the value U was very high, averaging 24, well over twice that for lymphoma and entirely in confirmation of Warburg's findings. The highest U value was 40.4 in a rapidly growing, highly malignant epidermoid carcinoma (S-28-2468). There seemed, indeed, to be some general correspondence between the value U and the degree of malignancy. Thus, S-29-1049, S-28-168J, and S-28-2468 all had a U value above 35 and were all highly malignant, while S-28-2524 and S-29-1042 were relatively benign, slow growing tumors, and each had a value below 11. But the rule was by no means invariable. Roughly the same crude parallelism could be traced between malignancy and anaerobic glycolysis, though again there were certain exceptions.

Sarcoma.—In all, thirteen cases of sarcoma were studied. In most cases the tumors investigated were primary foci and not metastatic in lymph nodes. The metabolism was striking and interesting, in that all figures were low. The average aerobic glycolysis was 0.017 mg., with comparatively little variation except in S-29-1803, where the figure was 0.036 mg. The average anaerobic glucose destruction was 0.037 mg., again varying closely about the mean. The U value averaged only 4.9 and the oxygen consumption only 2.4 c. mm. It is be noted that all these figures are far below those of carcinoma. To be sure, S-28-1803 with a U of 12.6 comes into the malignant class, but the majority fell far short of it and yet they were all highly malignant tumors. S-29-2965, a melanotic sarcoma, had an anaerobic glycolytic rate of but 0.056 mg. with a U of but 7.4, yet it was one of the most rapidly growing tumors ever seen in this laboratory. The conclusion is obvious. Sarcomata do not appear to behave metabolically in the same manner as do carcinomata, and from their relatively low metabolic rates it is difficult to understand wherefrom they derive the great energy which must be required to favor their growth and

advance. If one figures, according to the method of Hawkins (1925) the energy requirements of a rapidly growing carcinoma such as S-29-1049, one finds that it produces anaerobically 0.093 calories per mg. dry weight per hour, aerobically 0.206 calories. The same calculations applied to such an extremely rapidly growing sarcoma as S-29-2965 show that anaerobically it produces but 0.020 calories and aerobically only 0.022 calories. Again the distinction, metabolically speaking, between carcinomata and sarcomata is obvious. They do not behave in the same manner. It should be noted, however, that even carcinoma itself does not appear in such experiments as these to expend much energy in growing. Aerobically the average energy output of these series of carcinomata was 0.033 calories per mg. hour, anaerobically 0.054 calories. The respective averages for sarcomata were 0.018 and 0.014 and for benign tumors 0.049 and 0.006.

Benign Tumors.—Three benign tumors were investigated. As was to be expected, their metabolism was low, the aerobic glycolysis being 0.010 mg., and the anaerobic 0.017 mg., and the U 3.9—figures, it should be noted, not far from those of the highly malignant sarcoma. Their oxygen consumption averaged 2.4 c. mm.

The degree of differentiation of various cells seemed rather definitely connected with the degree of difference between the aerobic and anaerobic glycolysis.

For instance, Cases 9, 19, 33, 38, and 40 were tumors (carcinoma, lymphoma, etc.) composed of undifferentiated cells, and their percentage difference was only 1 per cent, the highest being 8 per cent. On the contrary, Cases 1, 8, 11, 12, 15, 16, 21, 30, 37, 41, 42, and 78 were all tumors composed of well differentiated cells, and the average difference was 167 per cent. Case 12 (S-28-2431) constituted an exception in that it was a highly differentiated tumor (epidermoid carcinoma) with a split of only 11 per cent. No correspondence was found to exist between the oxygen consumption and the maturity of the cells, though Glover, Daland and Schmitz (1930) have found with white blood cells of both the myeloid and lymphoid series a definite increase of oxygen consumption with increasing maturity. This they found to hold true only when the cells were studied in whole blood but not when they were suspended in Ringer's solution. For various reasons they believed that whole blood gave more accurate information. Exceptions to this general rule (the greater the difference between

aerobic and anaerobic glycolysis the greater the degree of cell differentiation) occurred in almost all the sarcomas, as witness S-29-1913, a highly differentiated tumor with a split of 0, and S-28-2721, an undifferentiated cell tumor with a split of 127 per cent.

DISCUSSION

It was hoped that evidence might be thrown on the question as to whether the malignant lymphomata are of infectious or neoplastic origin from this study of the aerobic and anaerobic glycolysis and the oxygen consumption of 71 lymph nodes and tumors. Careful comparisons have been made between the microscopic pathology and the metabolism. The various metabolic rates were found to vary over so large a range that few generalizations can be made. There was, in general, a steady rise in both the aerobic and anaerobic glycolysis from the normal nodes through Hodgkin's disease, the lymphoblastomata and tuberculous nodes to carcinomata. The sarcomata and benign tumors had very much lower rates. Their glycolysis, indeed, is not even of the same order of magnitude as that of the carcinomata. Much overlapping existed, however, so that from the metabolism alone one can not infallibly predict in which class the tissue will fall. Some lymphomata, for instance, had metabolic rates greater than those of some cancers. The oxygen consumption seemed to bear little or no relation to the pathological picture. It was rather consistently low in carcinoma and tended to be high in tuberculosis and some cases of Hodgkin's disease. The value U was over twice as high in the carcinomata as in any other pathological class, but here again overlapping existed to a considerable extent so that a diagnosis of neoplasm could not be made on this basis alone. The U value for sarcoma was definitely low, in no way approaching that of other malignant disease. The high metabolic rates found for tuberculous nodes indicated very clearly that the type of cell, rather than the nature of the underlying process, determined the character of the chemical changes. This fact was further borne out by the figures found for the rabbit lungs filled with mononuclears. Neither speed of growth nor degree of malignancy could be predicted from the glycolysis or oxygen consumption.

The metabolic processes in the sarcomata were of unusual interest. The figures of our thirteen cases approached those of benign tumors and were in no way similar to those of carcinomata. Yet some of these

sarcomata were extremely malignant and rapidly growing. The number studied is small and it is quite possible that further investigations will show that some human sarcomata have as high or even higher metabolic rates than do carcinomata of equal malignancy. Such is not our finding at present and further observations on sarcomata are in progress.

Such metabolic rates as are reported here represent the chemical activity of the tissue during a relatively brief period of tissue existence. They do not necessarily represent the requirements of growth. One of the essential characteristics of neoplastic tissues is their unlimited, chaotic and purposeless growth. To study the chemistry of growth rather than that of mere existence one must determine the metabolic rates of tissues over a long period of time in tissue cultures. This is being done as a continuance of the present study.

CONCLUSIONS

From a study of the metabolism of 71 lymph nodes and tumors one may conclude:

1. The nature of a tumor can not be predicted from the metabolism because too much overlapping of metabolic rates exists between the pathological groups.
2. There is no evidence metabolically one way or another as to whether malignant lymphomata of any type should be classed as neoplastic or as infectious processes.
3. The degree of cell differentiation can in most cases be foretold by the percentage difference between the aerobic and the anaerobic glycolysis. The greater the differentiation the greater the percentage difference. Sarcomata in general constitute an exception to this rule.
4. The degree of malignancy in carcinoma, but not in other tumors, can, with certain exceptions, be predicted from the height of the value U .
5. Human sarcomata appear to have a metabolism far more closely comparable to that of benign tumors than to that of carcinomata. They do not behave as malignant tumors under the Warburg classification. Their energy requirements are not of the same order as those of carcinoma.
6. One can not from the value U or from the glycolytic rates predict whether or not a tissue should be classed as neoplastic.
7. Warburg's findings for carcinomata are confirmed and amplified.

TABLE I

No.	Path. No.	Per cent active tissue	*Aerobic glycolysis	*Anaerobic glycolysis	†Oxygen consumption	"U"	Aerobic-anaerobic split per cent	Diagnosis
1	28-168J	50	0.116	0.172	3.2	36.6	48	Carcinoma
2	28-157R	100	0.071	0.117	6.4	16.4	65	Rabbit lung mono-nuclears
3	28-158R	100	0.050	0.106	5.2	16.1	110	" "
4	28-159R	100	0.051	0.100	5.1	15.0	96	" "
5	28-1686	100	0.034	0.037	2.0	4.5	9	Lymphoma
6	28-1804	100	0.066	0.082	4.4	11.7	24	Hodgkin's
7	28-2093	100	0.041	0.062	4.6	5.8	46	Hodgkin's
8	28-2347	100	0.009	0.014	1.0	2.5	56	Carcinoid
9	28-2348	100	0.069	0.069	5.0	7.5	0	Hodgkin's
10	28-2370	100	0.024	0.033	0.6	6.5	37	Normal node
11	28-2385	100	0.017	0.028	2.3	2.4	65	Arachnoidal fibroblastoma
12	28-2431	50	0.140	0.156	6.4	20.6	11	Carcinoma
13	28-2454	100	0.096	0.126	9.5	12.2	31	Tuberculosis
14	28-2467	100	0.055	0.082	1.8	17.9	51	Hodgkin's
15	28-2468	60	0.087	0.198	4.5	40.4	127	Carcinoma
16	28-2475	30	0.114	0.138	2.1	30.3	21	Carcinoma
17	28-2494	100	0.078	0.100	7.5	10.0	28	Hodgkin's
18	28-2516	100	0.040	0.069	4.4	8.4	73	Tuberculosis
19	28-2524	30	0.096	0.096	8.4	7.2	0	Carcinoma
20	28-2554	100	0.026	0.044	4.8	1.4	70	Endothelioma
21	28-2596	100	0.028	0.051	3.7	5.3	82	Normal nodes
22	28-2611	60	0.054	0.075	7.8	3.1	40	Reticulum cell sarcoma
23	28-2625	100	0.050	0.090	5.5	11.5	80	Hodgkin's
24	28-2665	100	0.067	0.096	6.4	11.2	43	Hodgkin's
25	28-2703	100	0.049	0.056	5.3	3.6	16	Hodgkin's
26	28-2707	100	0.019	0.040	2.1	5.8	110	Meningioma
27	28-2721	100	0.014	0.032	1.4	5.2	127	Sarcoma
28	28-2758	50	0.070	0.123	7.0	16.8	61	Carcinoma
29	28-2775	100	0.014	0.024	2.8	0.4	71	Fibrosarcoma
30	28-2795	100	0.056	0.078	5.1	9.3	39	Reticulum cell sarcoma
31	28-2917	75	0.076	0.082	3.7	13.6	8	Carcinoma
32	28-2931	100	0.077	0.106	6.8	11.7	35	Hodgkin's
33	28-3075	100	0.019	0.042	2.4	5.7	121	Normal node

* Expressed in mg. per 10 mg. dry weight per hour.

† " " c. mm. per mg. dry weight per hour.

TABLE I—*Concluded*

No.	Path. No.	Per cent active tissue	*Aerobic glycolysis	*Anaerobic glycolysis	Oxygen consumption	"U"	Aerobic-anaerobic split per cent	Diagnosis
34	29-100A	100	0.090	0.093	12.5	-1.7	3	Lymphoma
35	29-236	100	0.083	0.206	1.8	47.9	149	Lymphoma
36	29-266	20	0.115	0.115	4.5	17.8	0	Carcinoma
37	29-365	100	0.059	0.075	2.5	13.8	27	Lymphoma
38	29-399	100	0.044	0.045	0.7	9.8	2	Lymphoma
39	29-585	100	0.022	0.095	1.6	20.5	330	Lymphoma
40	29-628	100	0.001	0.012	4.4	-8.5	1000	Glioma
41	29-639	100	0.010	0.090	11.8	-1.1	800	Normal node
42	29-640	100	0.030	0.054	4.7	4.1	64	Hodgkin's
43	29-662	100	0.077	0.136	8.6	16.8	77	Lymphoma
44	29-1027	100	0.019	0.074	4.0	10.5	290	Tuberculosis
45	29-1042	75	0.032	0.048	2.0	10.0	50	Carcinoma
46	29-1049	50	0.150	0.250	29.5	35.5	67	Carcinoma
47	29-1152	100	0.047	0.100	2.7	19.6	112	Hodgkin's
48	29-1279	100	0.100	0.100	6.4	12.2	0	Lymphoma
49	29-1556	100	0.025	0.031	3.4	0.9	32	Hodgkin's
50	29-1574	100	0.071	0.071	6.4	4.9	0	Lymphoma
51	29-1585	100	0.047	0.047	4.8	3.4	0	Lymphoma
52	29-1754	100	0.068	0.079	9.7	0.3	14	Hodgkin's
53	29-1778	100	0.059	0.105	2.3	20.9	78	Carcinoma
54	29-1802	100	0.047	0.057	4.3	5.6	21	Hodgkin's
55	27-1803	100	0.036	0.077	3.3	12.6	114	Fibrosarcoma
56	29-1834	100	0.043	0.068	8.9	-0.9	58	Hodgkin's
57	29-1913	100	0.016	0.016	1.2	2.9	0	Fibrosarcoma
58	29-2066	33	0.042	0.168	3.9	34.2	300	Carcinoma
59	29-2309	20	0.050	0.200	10.0	30.0	300	Carcinoma
60	29-2368	100	0.032	0.067	5.3	6.1	110	Hodgkin's
61	29-2636	100	0.041	0.054	4.9	1.6	32	Hodgkin's
62	29-2651	100	0.046	0.065	6.5	3.3	41	Hodgkin's
63	29-J-1	100	0.014	0.024	1.9	2.2	41	Osteogenic sarcoma
64	29-J-2	100	0.010	0.015	0.8	2.1	50	Sarcoma
65	29-2866	100	0.030	0.060	2.3	10.4	100	Fibrosarcoma
66	29-2965	100	0.024	0.056	3.3	7.4	133	Melanotic sarcoma
67	30-396	100	0.018	0.060	3.9	7.2	232	Myxofibrosarcoma
68	30-600	100	0.062	0.120	10.4	9.2	94	Lymphoma
69	30-682	100	0.011	0.027	1.2	4.3	60	Fibrosarcoma
70	30-768	100	0.061	0.108	12.6	30.0	77	Lymphoma
71	29-664	50	0.012	0.046	9.6	-7.7	28	Melanotic sarcoma

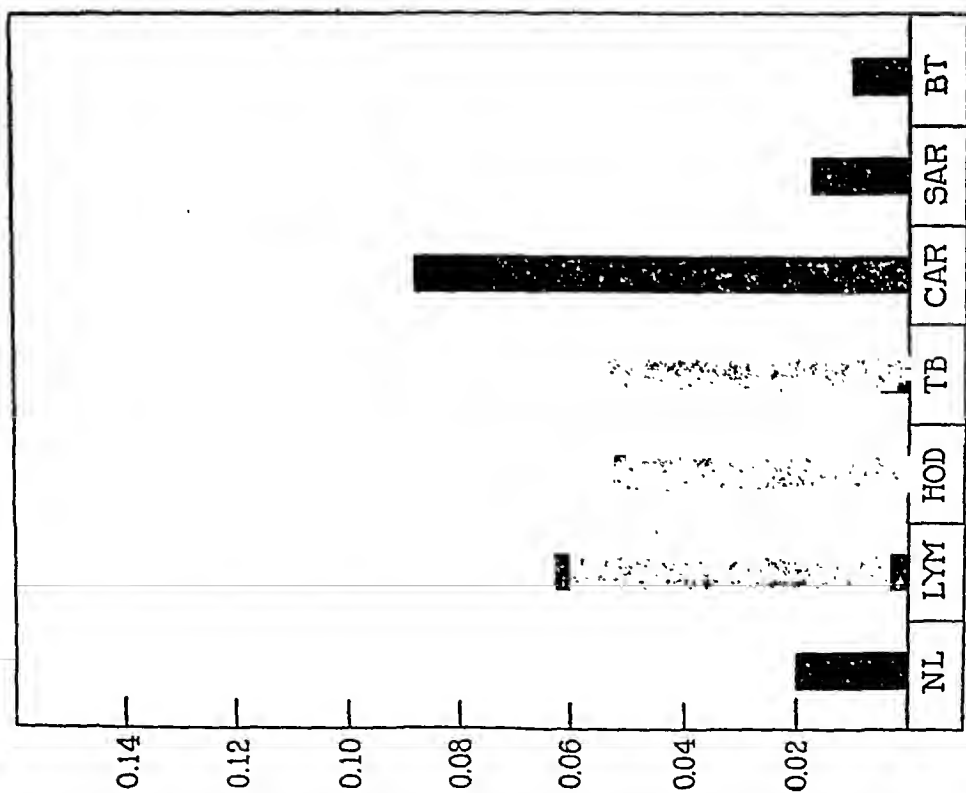


CHART 1

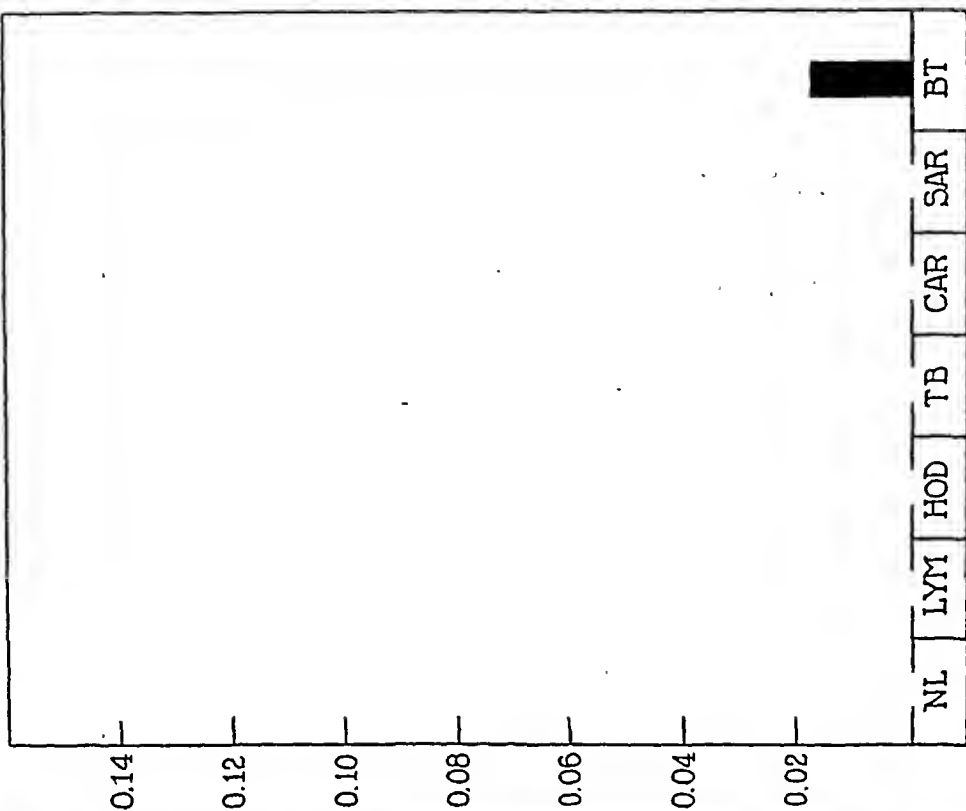
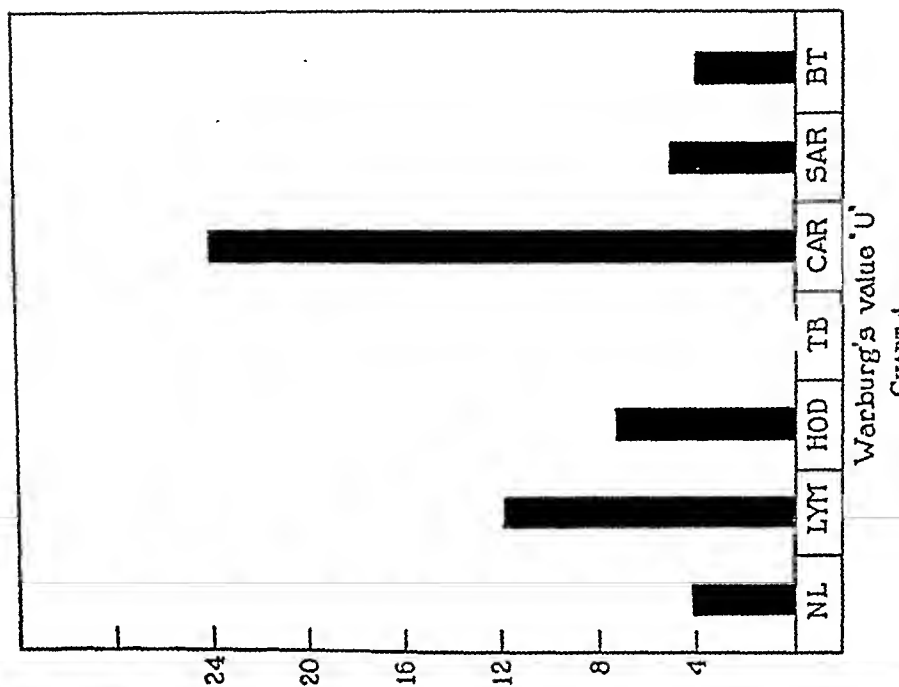
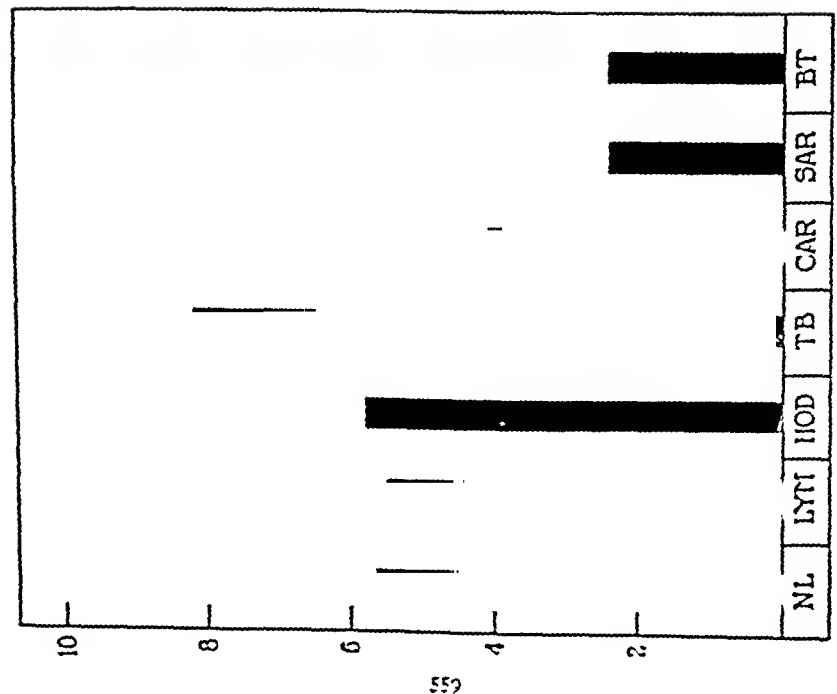


CHART 2



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SEROLOGICAL REACTIONS IN PNEUMONIA WITH A NON-PROTEIN SOMATIC FRACTION OF PNEUMOCOCCUS*

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It has been shown (1) that pneumococci contain two constituents which are chemically and antigenically distinct. One of these, the type-specific component, is a complex polysaccharide, predominantly present in the capsule of the organism; the other, a substance common to the pneumococcus species, is the so-called nucleoprotein, contained for the most part in the body of the cell. That these two chemically distinct fractions are responsible for the production of two qualitatively different antibodies has been demonstrated (1, 2).

The present report is based upon observations made with a third fraction derived from pneumococci and chemically distinct from both type-specific capsular polysaccharide and non-type-specific somatic nucleoprotein. For purposes of reference this substance is designated Fraction C. The chemical nature of Fraction C and the method of purification together with certain experimental observations are presented in a separate communication (3). In this report it is sufficient to state that Fraction C is a non-protein material of somatic origin and appears to be a carbohydrate common to the pneumococcus species. Although final proof of its exact nature rests upon chemical analysis, nevertheless convincing evidence of the separate identity of Fraction C is brought out by the serological reactions to be described.

Material and Methods

Preparation of Fraction C.—The material employed in the serological tests was derived from a degraded, non-type-specific R strain of *Pneumococcus*. A strain of this character was employed in order to minimize the presence of type specific carbohydrate. Fraction C was obtained in the following manner: The organisms

* Presented before the American Society for Clinical Investigation at a meeting held in Atlantic City, May 5, 1930.

contained in several liters of full-grown broth culture of an R strain were centrifuged and resuspended in normal salt solution in 60 to 100-fold concentration. The bacteria were then frozen and thawed several times until dissolution had been effected. 0.3 cc. to 0.5 cc. of normal acetic acid was added and the solution boiled for 8 to 10 minutes. The heavy coagulum thus formed was removed by centrifugation. Acidulation and boiling were repeated to insure removal of all acid and heat precipitable material. The final water-clear supernatant fluid, neutralized with normal NaOH, contained Fraction C. Material prepared according to this simple procedure was comparable in reactivity and specificity to more highly purified preparations. Consequently, further steps in purification were not, as a rule, carried out. Some lots, however, were treated by repeated precipitation with 4 to 5 volumes of 95 per cent alcohol.

In the charts the reaction is represented as occurring in dilutions of C substance as high as 1 to 640,000. This figure is only approximate since a quantitative estimation was not made on all lots of material. However, accurate measurement was made with one sample; using it as a standard some of the sera were re-tested and found to react with a 1 to 1,000,000 dilution. Consequently the approximate figures given are believed to be conservative estimates.

The chemical fractionation of numerous bacteria by procedures similar in many respects to that just described, has been reported by others. Pick (4) derived from typhoid bacilli an apparently non-protein substance which reacted especially in immune serum. Zinsser (5) and Zinsser and Parker (6) isolated so-called residue antigens from tubercle bacilli, pneumococci, staphylococci, influenza bacilli, and typhoid bacilli. Laidlaw and Dudley (7) obtained a carbohydrate from tubercle bacilli. Furth and Landsteiner (8) obtained from typhoid and paratyphoid B cultures three fractions, one of which appeared to be non-protein. Day (9) derived from staphylococci an "antigenic specific substance." Lancefield (10) separated a somatic carbohydrate from hemolytic streptococci which she designated Fraction C and which is comparable in many respects to the pneumococcus Fraction C.

Pneumococcus Type-Specific Polysaccharides.—The preparations employed were made according to the method described by Heidelberger and Avery (11).

Pneumococcus Nucleoprotein.—The material was made by precipitation with acetic acid in the cold as described by Avery and Morgan (12).

Precipitin Tests.—0.2 cc. test serum was diluted to 0.5 cc. with physiological salt solution; to this was added an equal volume of varying dilutions of precipitogens. Final readings were made after the mixtures had been kept in water bath at 37° for 2 hours and over night in the ice box.

Agglutination Tests.—0.5 cc. of varying dilutions of serum was added to 0.5 cc. of heat-killed pneumococci suspended in physiological salt solution. The tubes were incubated at 37° for 2 hours and allowed to stand overnight in the ice box before final reading was made.

Sera obtained at frequent intervals from patients acutely ill with pneumonia or convalescent from the disease have been mixed with varying dilutions of Fraction C and the presence or absence of precipitation noted. Charts 1 and 2 illustrate results which have been repeatedly obtained.

Chart 1 gives the results of observations in a case of Type II pneumococcus pneumonia. Serum obtained on admission to the hospital in the third day of disease reacted with high dilutions of Fraction C.

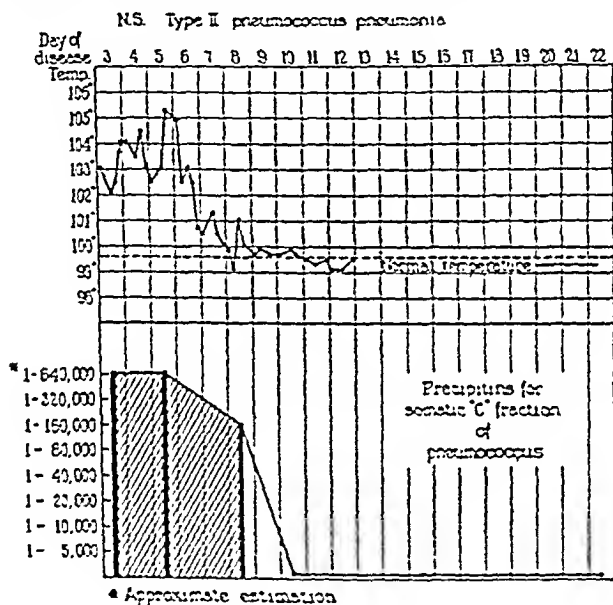


CHART 1

This reactive capacity dropped slightly on the day of crisis and completely disappeared in the next 2 days.

Chart 2 presents a similar course of events obtained with serum derived from a patient suffering from Group IV pneumococcus pneumonia. The phenomenon exhibited in Charts 1 and 2 has been observed in each of 50 patients ill with pneumococcus pneumonia. In each instance it has been found that serum obtained during the acute stage possesses a high titre of precipitins for Fraction C. A day or two after recovery this precipitating power is no longer detectable. The occurrence of the phenomenon is unrelated to the type of Pneu-

mococcus causing infection. The fact that serum obtained from patients on admission possessed, in every instance, anti-C precipitins is evidence of the early appearance of the reactivity. The serum obtained from one patient as early as 18 hours after the initial chill reacted with high dilutions of C substance. Individuals who succumbed to the disease maintained C precipitins until death. The age of patients, whose sera have been tested, ranged from 7 years to

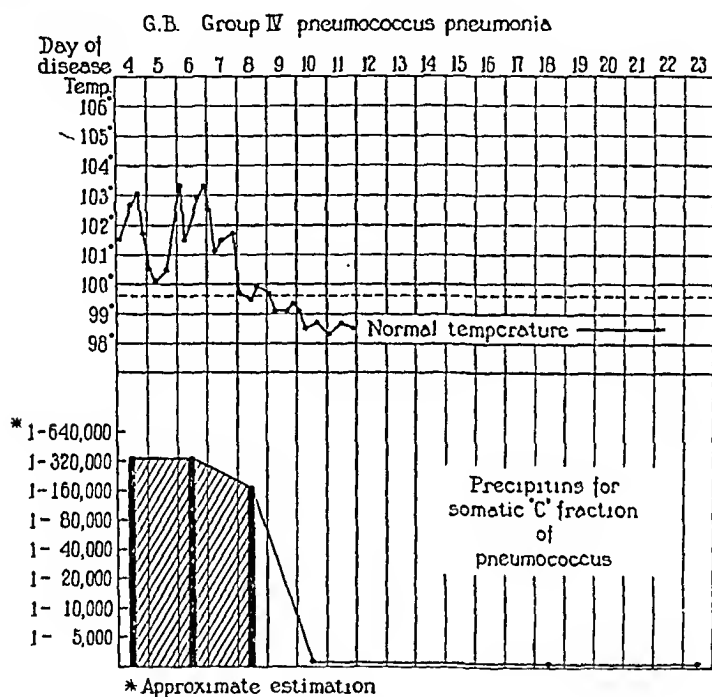


CHART 2

65 years; the phenomenon was present in the youngest and oldest. Sera from normal individuals have not been found to possess demonstrable precipitins for Fraction C.

The observations on the serological reactivity of pneumococcus Fraction C presented in this report are limited to precipitin tests made with sera from patients. However, it may be mentioned that antipneumococcus sera derived from animals, in some instances, precipitate Fraction C. This is particularly demonstrable with the antipneumococcus horse sera¹ used for typing of pneumococci. The

¹ The antipneumococcus horse sera were obtained through the courtesy of Dr. A. B. Wadsworth from the New York State Board of Health Laboratories, Albany, New York.

available lots of antisera of Types I, II and III all precipitate Fraction C, Type III serum apparently reacting in highest titre. Of sera derived from 24 rabbits immunized with either Types I, II, III, or R pneumococci, only 3 possessed demonstrable anti-C precipitins; each of these animals had received heat-killed

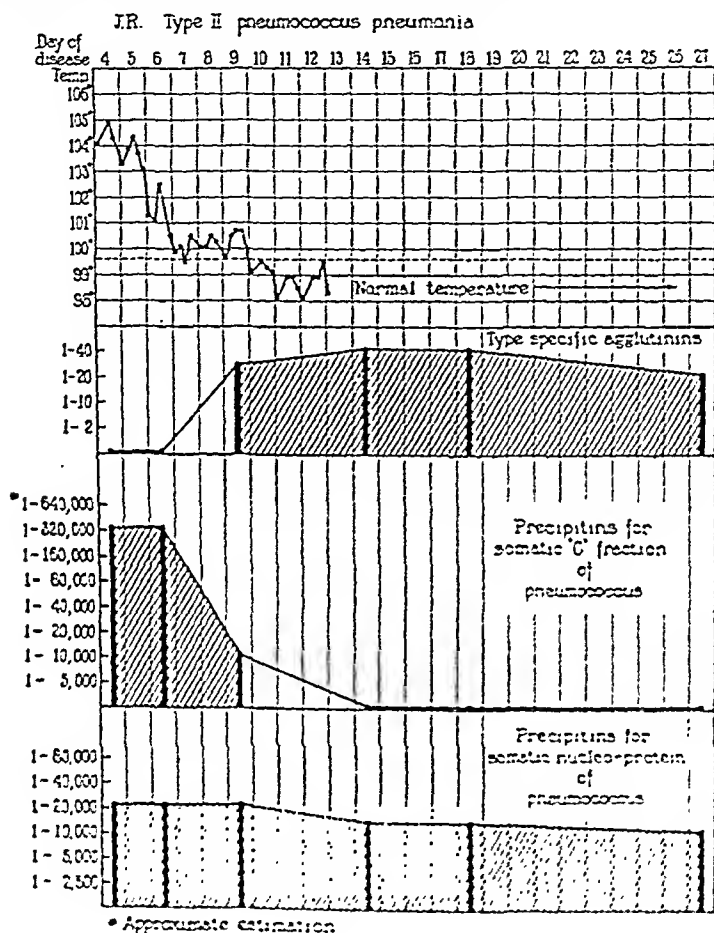


CHART 5

cultures of Type III *Pneumococcus*. At the present time the conditions which favor the production experimentally of anti-C antibodies are not understood.

For purposes of comparison, sera derived from patients during the course of pneumonia were tested for the presence of type-specific

antibodies and for pneumococcus protein antibodies as well as for C precipitins. The results when charted in relation to the course of pneumonia, present three different curves of antibody titre. Observations of this character are presented in Chart 3.

From Chart 3 it will be seen that type-specific antibodies are absent during the acute illness and are first demonstrable at the time of crisis, whereas precipitins for C substance are present in high titre during the febrile stage and are no longer detectable a few days after

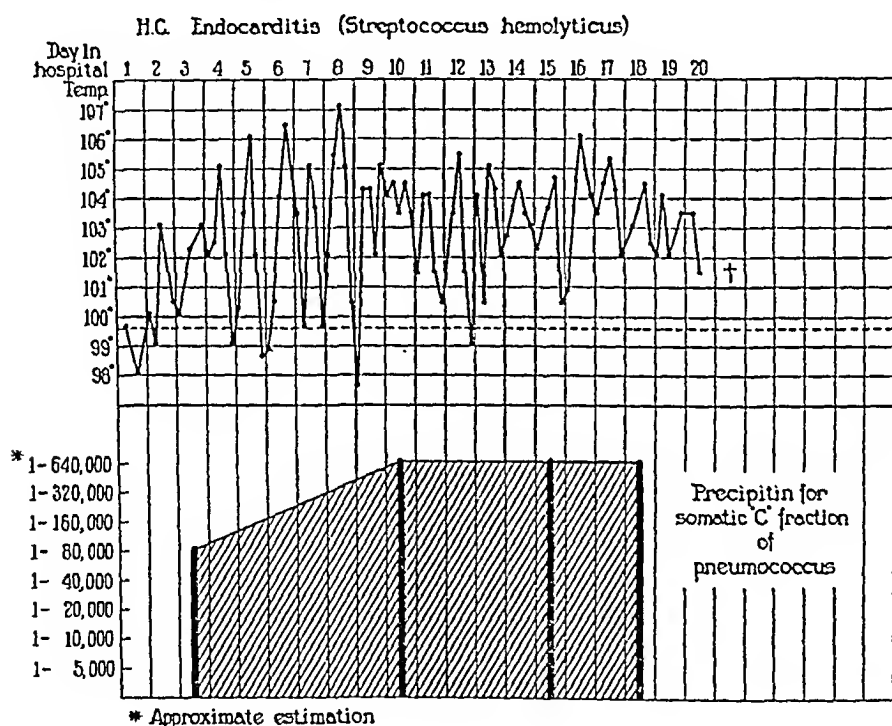


CHART 4

the critical fall in temperature. Antiprotein antibodies do not vary markedly during the course of illness, although there seems to be a slight reduction in titre as convalescence progresses. Observations similar to those given in Chart 3 have been made on twenty cases of pneumonia. It is an interesting fact, therefore, that with three chemically distinct fractions derived from the same bacterial cell, serological reactions involving three qualitatively different antibodies may be demonstrated during the progress of pneumococcus pneumonia.

Up to this point the report has been limited to the results obtained with the sera of patients suffering from pneumococcus infection. A limited number of individuals acutely ill with other febrile diseases have been available for comparative tests. Four cases of hemolytic streptococcus infection ending fatally were observed from admission to the hospital until death. Three of these had streptococcus pneumonia. In each instance their sera precipitated pneumococcus Fraction C equally as well as did sera from cases of pneumococcus pneumonia. The fourth patient had malignant endocarditis; hemolytic

5. Acute rheumatic fever

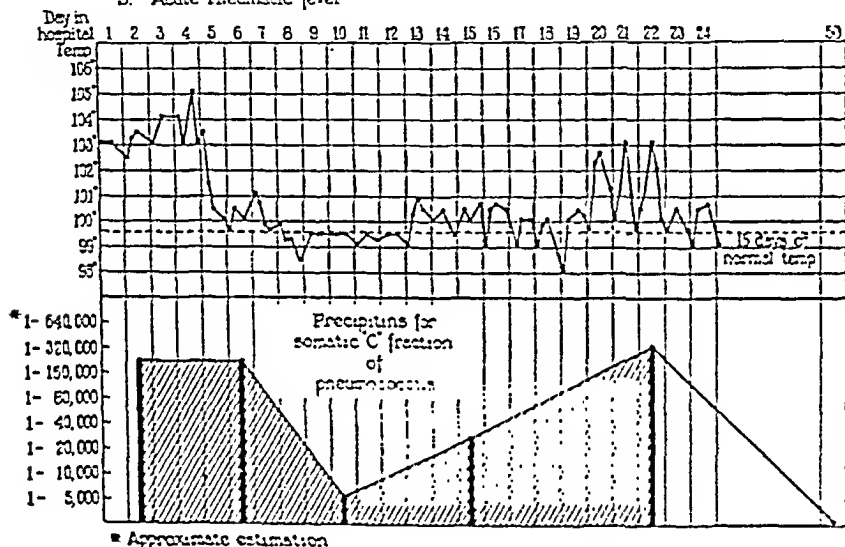


CHART 5

streptococci in large numbers were repeatedly demonstrated in blood cultures. The result of serological tests made with pneumococcus C substance in this case is presented in Chart 4.

As can be seen from Chart 4 the patient's serum maintained throughout the illness the capacity to react in high titre with C substance of *Pneumococcus*.

Fifteen cases of acute rheumatic fever have been followed by similar serological tests through stages of active disease and remission. Chart 5 records the results obtained in one of this group.

As demonstrated by Chart 5 the course of the temperature and the capacity of the patient's serum to precipitate pneumococcus Fraction C closely parallel each other. The same relations were found to hold in the other fourteen cases of rheumatic fever. All of the patients at the time of elevated temperature exhibited symptoms and signs of active disease.

TABLE I

Reactions with Fraction C in Febrile Diseases Other than Pneumococcus Pneumonia

Patient	Disease	Temperature	*Dilutions of Fraction C		
			1-10,000	1-80,000	1-120,000
Das.....	Malaria	104°	—	—	—
Mek.....	Lung abscess	102.5°	+	++	+++
Fol.....	Cirrhosis of liver	103°	—	—	—
Mit.....	Typhoid fever?	104°	—	—	—
Rid.....	Pericarditis, rheumatic origin	103°	+	++	++
Reg.....	Unexplained anaemia	104°	—	—	—
Hag.....	Tuberculosis of lungs	102.5°	±	—	—
Mer.....	Unexplained fever—Tbc.?	103.5°	—	—	—
Hin.....	Osteomyelitis (staphylococcus)	105°	+++	+++	++
McD.....	Measles	102.8°	—	—	—
Pe.....	Chicken pox	103°	—	—	—
Mac.....	Endocarditis (<i>Streptococcus viridans</i>)	104°	+	+	±

* Approximate estimation.

In addition to the hemolytic streptococcus infections and cases of acute rheumatic fever just mentioned, single tests were made on sera obtained in a miscellaneous group of diseases. The results are listed in Table I.

From Table I it can be seen that patients, whose sera precipitated pneumococcus Fraction C, were in each instance suffering from disease associated with Gram positive cocci. The number of cases so far available is too few to attach undue significance to this point. However, the results suggest that etiological agent and serological reactivity with Fraction C may have some causal relationship.

DISCUSSION

The observations recorded in this communication have been made with a constituent of pneumococcus cells which is chemically distinct from both the type-specific capsular carbohydrate and the somatic nucleoprotein. Although pneumococcus Fraction C in all probability is a nitrogenous sugar, it differs from the soluble specific substance in that it possesses no type-specificity and is contained, as a common species constituent, within the body of the cells. Pneumococcus Fraction C appears to be analogous to the somatic carbohydrate isolated from hemolytic streptococci by Lancefield (10).

By testing sera obtained from cases of lobar pneumonia during the progress of the disease two interesting observations were made. The first of these is the early appearance of precipitins for C substance and the second is the complete disappearance of the phenomenon 1 to 3 days after crisis. Sera obtained from patients on the day of admission to the hospital have in every instance reacted with Fraction C. This was true even of cases admitted in the first 24 hours of disease. Since the serum of no normal individual has exhibited this reaction, the appearance of C precipitins is associated closely with the onset of pneumonia. Although an explanation of the phenomenon is not yet available, a possible interpretation is suggested by the so-called "anamnestic" reaction. Cole (13) found that in an animal which had previously been immunized and in which the antibodies in the blood had disappeared a subsequent injection of the original antigen caused antibodies to reappear more rapidly than did the primary injections. Bieling (14) employing two antigens (*B. dysenteriae* and *B. typhosus*), found that the reinjection of one brought back heterologous as well as homologous antibodies. In the course of similar experiments McKenzie and Fröhbauer (15) using four antigens of remote biological origin were able to cause heterologous antibodies promptly to reappear by the injection of one antigen. Applying the principles involved in the "anamnestic" reaction to the observations recorded in this paper, it is possible that the occurrence of pneumococcus C precipitins early in pneumonia may be determined by a previous experience of the individual with some suitable bacterial antigen.

In addition to the observations with Fraction C, the investigation

was extended to include a more complete study of the occurrence during pneumonia of antibodies reactive with pneumococcus constituents. In these latter experiments, therefore, tests were made for antibodies reactive with: 1. Type-specific capsular polysaccharide; 2. Non-type-specific somatic polysaccharide; 3. Non-type-specific somatic nucleoprotein. By charting the result of these serological reactions with reference to the course of the disease, the occurrence of each of the qualitatively different antibodies was shown to follow a different course. It is an interesting fact, therefore, that during a *single* infection, the body responds in such a manner that antibodies reactive with different components of the causative organism become demonstrable at different stages of the disease. The results serve to illustrate the complexity of the immunological mechanism which presides over the response to bacterial infection. The observations recorded in this paper also demonstrate that the reactivity of a patient's serum with pneumococcus C substance is not specific for pneumococcus infection. Sera from a limited number of febrile diseases due to other causes have been tested. In this small group, precipitation of C fraction occurred most definitely in those instances where Gram positive cocci were proven to be or were suspected of being the etiological agent. If the implications contained in these few cases are substantiated by a larger number of tests the results suggest some, as yet undetermined, broad relationship existing among certain bacterial infections.

SUMMARY

1. Sera from individuals acutely ill with lobar pneumonia possess the capacity to precipitate in high titre a non-protein somatic fraction derived from pneumococci (Fraction C). Following crisis the reaction is no longer demonstrable.

2. Sera obtained from cases of pneumococcus pneumonia during illness and convalescence have been tested for antibodies specifically reactive with three chemically distinct constituents of *Pneumococcus*. The results, when correlated with the course of disease, demonstrate differences in the occurrence of each qualitatively distinct antibody.

3. The precipitation of pneumococcus Fraction C is not limited to the sera of individuals ill with pneumococcus infection. But in the

few other cases available for comparative tests, definite reactions have been obtained only in streptococcus and staphylococcus infections and in acute rheumatic fever.

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CUTANEOUS REACTIONS IN PNEUMONIA. THE DEVELOPMENT OF ANTIBODIES FOLLOWING THE INTRADERMAL INJECTION OF TYPE-SPECIFIC POLYSACCHARIDE

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In a previous communication (1) the occurrence of cutaneous reactions to the polysaccharides and proteins of *Pneumococcus* during lobar pneumonia was reported. The investigation disclosed the interesting fact that the capsular carbohydrates of *Pneumococcus* were capable of inciting a reaction when injected intradermally. The character of the response and the conditions under which it was obtained are briefly summarized as follows: The characteristic reaction is the development of an *immediate wheal and erythema* at the site of injection; a positive reaction was first elicited at or about the time of crisis; it was produced only by the polysaccharide homologous in type to that of the *Pneumococcus* causing the disease; at the time of reaction type-specific antibodies for the homologous organism were demonstrable in the patient's serum.

In contrast to the polysaccharides, the so-called nucleoprotein fraction of the *Pneumococcus* evoked a *delayed* response which reaches its height in 24 hours. It simulated in appearance the tuberculin reaction. The protein reaction generally appeared first early in convalescence and tended to become progressively more marked during the course of recovery. Circulating antibodies for the nucleoprotein were present in approximately equal concentrations during the periods of acute illness and recovery. The protein reaction bore no specific relation to the type of *Pneumococcus* producing the infection.

The present report includes further observations on cutaneous reactions to pneumococcus fractions and deals especially with the appearance of antibodies for more than one specific type of *Pneumococcus*

in the serum of convalescent patients. The individuals whose sera presented these unusual reactions had received repeated intradermal injections of the pneumococcus carbohydrates. The possible relationships between the repeated injection of the specific substances and the antibody formation will be discussed.

Materials and Methods

A. Skin Test Materials

1. *Type-Specific Capsular Polysaccharides*.—The type-specific polysaccharides were obtained in purified form from Types I, II and III *Pneumococcus* by the method of Heidelberger and Avery (2). They are chemically and serologically distinct and have been identified with the capsular material of the cell. The Type II and Type III polysaccharides are nitrogen-free; that of Type I contains 5 per cent nitrogen as part of the sugar molecule (3). The polysaccharides were diluted in physiological salt solution to a concentration of 1:10,000 and 0.1 cc. containing 0.01 mg. of the specific soluble substance was injected intracutaneously. Skin tests were repeated at intervals throughout the disease and convalescence.

2. *Nucleoprotein*.—The nucleoprotein was prepared by the method of Avery and Morgan (4) from an R strain derived from a Type II S organism. The material was standardized on the basis of nitrogen content and 0.01 mg. of protein in 0.1 cc. of physiological saline was used in all tests. These tests were repeated at approximately weekly intervals during the patient's stay in hospital.

B. Titration of Sera for Antibodies

At intervals during the acute illness and convalescence samples of serum were obtained and tested for the presence or absence of circulating antibodies.

1. *Type-Specific Antibodies*.—The presence in the serum of type-specific antibodies was determined by (a) agglutination, and (b) passive protection of mice.

a. *Type-Specific Agglutinins*.—0.5 cc. of varying dilutions of the serum to be tested was mixed with 0.5 cc. of a heat-killed suspension of type-specific pneumococci. Final readings were made after the mixtures had been incubated for 2 hours in the water bath and then placed in the ice box overnight.

b. *Passive Protection of Mice*.—The capacity of patient's serum to protect mice against infection was tested by the usual technique. 0.2 cc. of serum diluted to 0.5 cc. with salt solution was employed. So far as possible each serum was tested against pneumococci of Types I, II and III. The cultures used were invariably fatal for mice in doses of 0.0000001 cc.

2. *Antibodies for Nucleoprotein*.—Antibodies for the nucleoprotein material were determined by the precipitin reaction as previously described (1).

A study of the cutaneous response to the injections of the type-specific polysaccharides of the Pneumococcus and of the occurrence of circulating antibodies has been made in 74 cases of lobar pneumonia. The results confirm earlier observations and may be briefly summarized as follows: Positive reactions were elicited only with the homologous polysaccharide at crisis or shortly thereafter; homologous type-specific antibodies were demonstrable in the serum of each reactive patient. In no instance was a positive skin reaction to a polysaccharide obtained in the absence of circulating antibodies for the type of Pneumococcus from which the polysaccharide was derived. Table

TABLE I
The Incidence of Cutaneous Reactions to the Homologous Polysaccharide in Convalescent Patients

Type of infecting organism	No. patients	No. positive reaction	Per cent giving positive reactions
I	21	21	100
II	17	10	58.8
III	9	4	44.4

13 recovered cases of Group IV—no reactions to polysaccharides of Types I, II, III.

5 recovered cases of Type II (atypical)—no reactions to polysaccharides of Types I, II, III.

9 fatal cases of various types—no reactions to polysaccharides of Types I, II, III.

I shows the relative frequency of positive reactions to the homologous type of polysaccharide in patients convalescent from Type I, II or III pneumonia. All but 4 of the Type I cases were treated with anti-pneumococcus Type I serum.

From Table I it can be seen that positive cutaneous reactions were obtained in all of the Type I cases and in approximately 50 per cent of the Types II and III patients. The polysaccharides of Types I, II and III elicited no response in the 13 Group IV and the 5 atypical Type II patients. The absence of reactivity in the 9 fatal cases agrees with earlier experiences in that in patients with lethal termination no reaction was obtained. 12 patients convalescent from Type II or Type III pneumonia also failed to react but in 8 of them no reaction

would be expected since their sera contained no circulating antibodies; the sera of the remaining 4 patients contained circulating antibodies but no reaction to the polysaccharide occurred. Thus, of the entire group of patients included in Table I a positive skin reaction was expected in 39 by virtue of the fact that they were recovered cases of Type I, II or III infection whose sera contained type-specific antibodies. Positive reactions were obtained in 35 of them.

The cutaneous response to the nucleoprotein fraction of the *Pneumococcus* was studied in the same group of cases. The results have confirmed the validity of the earlier conclusions. The delayed protein reaction appeared first during convalescence. The reactivity of the patient did not appear to be influenced by the titer of circulating antibodies for the protein fraction.

Occurrence of Antibodies to Heterologous Types of Pneumococcus

Other observers have repeatedly demonstrated the occurrence of circulating antibodies for the homologous type of *Pneumococcus* at or about the time of crisis. The present investigation confirms these results and, in addition, discloses the fact that at the time when type-specific antibodies are first demonstrable in the blood the patient becomes reactive to the cutaneous injection of the homologous type-specific polysaccharide. The reactions have been specifically limited to the homologous types. In the present study repeated tests were made with the type-specific polysaccharides of Types I, II and III with a view to determining the length of time through which the reactive capacity might persist. In view of the fact that positive results at the time of recovery were strictly limited to the homologous type, it was rather surprising to observe after repeated tests that a positive skin reaction was obtained in certain instances with a polysaccharide of heterologous type as well. With the appearance of the skin reaction to the heterologous polysaccharide, circulating antibodies for the heterologous type of *Pneumococcus* were demonstrable in the patient's serum, in addition to those for the homologous type. The chief interest in this report, therefore, is concerned with the development of specific antibodies for more than one type of *Pneumococcus* in patients who have repeatedly received intradermal injections of type-specific carbohydrates. These "additional" antibodies were

characterized by the fact that they were heterologous in type to that of the infecting organism and were not demonstrable at crisis but appeared 1 to 3 weeks after the patient's recovery. These features were disclosed as a result of what at first appeared to be a discrepancy in specificity of the cutaneous reaction. The results can be more clearly brought out by describing in detail the course of events in one patient.

M. A., a girl of 14 years, suffering from Type II pneumonia was admitted on the second day of disease. No circulating antibodies were demonstrable in the patient's serum at this time. Crisis occurred on the tenth day. On the thirteenth day skin tests with the polysaccharides of Types I, II and III revealed a positive reaction only to the Type II material. The serum taken shortly thereafter contained circulating antibodies for Type II *Pneumococcus* alone. 8 days after the first test the skin tests were repeated and at this time positive reactions occurred both to Type II and Type I polysaccharides. At this time her serum contained antibodies for Type I as well as for Type II *Pneumococcus*. (See Chart 1.)

Because of the appearance of heterologous antibodies and skin reactions a series of patients was studied in order to determine the frequency with which the phenomenon occurred. Skin tests and antibody determinations were made at varying intervals in 18 patients. Of these, 3 were cases of Type I pneumonia, 7 of Type II, 3 of Type III and 5 of Group IV. At or about the time of crisis antibodies for the homologous type of *Pneumococcus* appeared in all but 2 of the Types I, II and III patients. In the Group IV cases tests were not carried out with the homologous organism. However, in no instance were heterologous antibodies demonstrable at that time. As convalescence progressed, repetition of skin tests and antibody determinations revealed that there had *developed*, since they were previously absent, specific antibodies and a positive skin reaction for at least one of the heterologous types of *Pneumococcus*. Table II shows the intervals between the appearance of homologous and heterologous antibodies in relation to the day of disease and recovery.

From Table II it can be seen that in 10 of the 18 patients heterologous antibodies were detected during convalescence; in 3 Type I patients, 4 Type II, 2 Type III, and 1 of Group IV. In the great majority of cases the presence of circulating antibodies for the heterologous type was first indicated by a positive skin test to the polysac-

TABLE II

Differences in Time of Appearance of Homologous and Heterologous Antibodies in Patients Receiving Skin Tests with Type-Specific Polysaccharides

Patient	Antibodies to homologous type		Antibodies to heterologous types				Day of crisis
	Type	Day observed	Type			Day observed	
L. H. (serum), No. 7165.....	I	3			III	17	6
M. P. (serum), No. 7263.....	I	8		II		14	5
F. R., No. 7253.....	I	9		II		22	7
M. C., No. 7183.....	II	14	I			19	5
G. M., No. 7248.....	II	10	I			19	9
E. C., No. 7269.....	II	11	I			18	9
M. A., No. 7261.....	II	15	I			22	10
H. R., No. 7262.....	III	6		II		19 26	8
K. R., No. 7143.....	III	5	I	II		21	4
A. P., No. 7211.....	IV	Not determined	I		III	26 44	7
M. C., No. 7189.....	II	15	0	0	0		8
I. S., No. 7195.....	II	9	0	0	0		6
C. Q., No. 7225.....	II	0	0	0	0		7
J. F., No. 7171.....	III	0	0	0	0		10
G. B., No. 7178.....							8
R. F., No. 7256.....							9
T. R., No. 7197.....	IV	Not determined	0	0	0		5
G. R., No. 7236.....							8

charide of that type. The type of *Pneumococcus* for which heterologous antibodies developed was in 7 instances Type I, in 4 Type II, and in 2 Type III. In 3 cases specific antibodies for 2 heterologous types appeared. Although the homologous antibodies were detectable at the time of crisis, those for heterologous types were usually noted in the second week of convalescence, the earliest being 9 days after recovery, the latest 37 days after recovery. The period of time during which antibodies were detectable in the serum and skin reactivity remained positive varied from a few days to several months.

These observations led to the hypothesis that the mechanism involved in the production of antibodies for heterologous types of *Pneumococcus* was associated with the intradermal inoculations of the type-specific

TABLE III

The Incidence of Type-Specific Antibodies in the Control Series of Patients

Type of infecting organism	No. cases	Antibodies for homologous type of <i>Pneumococcus</i>	Antibodies for heterologous types of <i>Pneumococcus</i>
I	6	5	1*
II	6	6	1*
III	2	2	0
Group IV	7	Not determined	1**

* Type III antibodies present during disease and recovery.

** Type II antibodies present during disease and recovery.

polysaccharides. In order to obtain more information on this point the next 21 pneumonia patients admitted to hospital were studied as controls. In this series no injections of the specific polysaccharides were made but the serum was tested at intervals for circulating type-specific antibodies to *Pneumococcus* Types I, II and III. In the Group IV cases tests were not made for antibodies reactive with the infecting organism. In most of the 21 cases homologous antibodies were observed at the time of recovery. However, in striking contrast to the group of patients which had received repeated injections of the specific soluble substance, no patient in the control series *developed* antibodies for a heterologous type. In only 3 patients (1 each of Types I, II and Group IV) antibodies for a type other than that of the infectious agent were observed. In these three instances the presence

of the heterologous antibodies was detected in low titre early in the acute illness and the concentration of the antibodies did not increase during convalescence. They, therefore, bear no apparent relation to the present infection but are presumably attributable to a preexisting state. This interpretation is substantiated by the fact that some normal individuals possess in their serum type-specific antibodies.

Table III presents the distribution of the types of *Pneumococcus* in the control group of patients and the number in whom circulating antibodies were detectable.

The 21 patients who served as controls were comparable to the test group with regard to age incidence, distribution of types of infecting *Pneumococcus*, severity of the infection and period of observation.

The patients in whom heterologous antibodies were observed, received injections of 0.01 mg. of the type-specific capsular polysaccharides derived from the *Pneumococcus* of Types I, II and III. The average number of injections before the appearance of antibodies for the corresponding type of *Pneumococcus* was three. In 3 cases the antibodies were detected after a single injection. The largest total amount which was followed by heterologous antibody production was 0.05 mg. whereas in other cases heterologous antibodies did not develop even after the injection of as much as 0.08 mg. of the polysaccharides. These irregularities demonstrate the fact that the production of antibodies is not solely dependent upon the quantity of polysaccharides injected. The average time between the last injection and the appearance of the antibodies was 6 to 10 days but in one case they were first detected 20 days after the last injection. The exact data regarding the individual patients in whom antibodies for heterologous types of *Pneumococcus* developed are presented in graphic form in composite charts. These charts show in relation to the day of disease, the time of appearance of type-specific agglutinins and mouse protective antibodies for homologous and heterologous types of *Pneumococcus*, the time, number and results of skin tests with the 3 type-specific polysaccharides and the period during which the observations were continued.

DISCUSSION

The primary interest of the present work centers in a group of 18 cases of lobar pneumonia 10 of whom, in convalescence, developed

Type I

Name of patient	L.H. Age 30 (Serum) *7165	T.P. Age 31 (Serum) *7253	F.R. Age 32 (Serum) *7253
Day of disease	2 3 4 6 9 16 17 23 27 49	3 4 5 7 8 13 14 20 21	7 9 11 22 28 29 35 36
Skin tests	I - - - - - II - - - - - III - - - - -	I - - - - - II - - - - - III - - - - -	I - - - - - II - - - - - III - - - - -
Type specific agglutinins	I - - - - - II - - - - - III - - - - -	I - - - - - II - - - - - III - - - - -	I - - - - - II - - - - - III - - - - -
Protective antibodies	I - - - - - II - - - - - III - - - - -	I - - - - - II - - - - - III - - - - -	I - - - - - II - - - - - III - - - - -
Blood culture	+	+	-

Type II

Name of patient	T.C. Age 30 *7103	G.H. Age 47 *7240	E.C. Age 31 *7263	T.A. Age 14 *7261
Day of disease	2 3 5 6 10 14 19 21 25 30 33 37	6 8 9 10 13 19 20 25 26	2 9 10 11 17 18 24 25 31 32 34	2 10 13 15 21 22 29 33 35 42 50 59
Skin tests	I - - - - - II - - - - - III - - - - -	I - - - - - II - - - - - III - - - - -	I - - - - - II - - - - - III - - - - -	I - - - - - II - - - - - III - - - - -
Type specific agglutinins	I - - - - - II - - - - - III - - - - -	I - - - - - II - - - - - III - - - - -	I - - - - - II - - - - - III - - - - -	I - - - - - II - - - - - III - - - - -
Protective antibodies	I - - - - - II - - - - - III - - - - -	I - - - - - II - - - - - III - - - - -	I - - - - - II - - - - - III - - - - -	I - - - - - II - - - - - III - - - - -
Blood culture	-	-	-	-

Type III

Name of patient	H.R. Age 21 *7252	K.R. Age 22 *7143
Day of disease	6 8 9 10 12 13 19 25 26 30	2 4 5 7 8 10 12 20 21 28 32 41 74
Skin tests	I - - - - - II - - - - - III - - - - -	I - - - - - II - - - - - III - - - - -
Type specific agglutinins	I - - - - - II - - - - - III - - - - -	I - - - - - II - - - - - III - - - - -
Protective antibodies	I - - - - - II - - - - - III - - - - -	I - - - - - II - - - - - III - - - - -
Blood culture	-	-

Group IV

Name of patient	A.P. Age 35 *7211
Day of disease	5 7 8 12 13 18 19 20 26 44
Skin tests	I - - - - - II - - - - - III - - - - -
Type specific agglutinins	I - - - - - II - - - - - III - - - - -
Protective antibodies	I - - - - - II - - - - - III - - - - -
Blood culture	-

CHART 1. The development of heterologous antibodies in patients receiving intradermal injections of type-specific polysaccharides.

circulating antibodies for at least one type of *Pneumococcus* heterologous to that etiologically related to the disease. All of the patients in whom the phenomenon was observed had received repeated intradermal injections of 0.01 mg. of specific capsular polysaccharide from each of Types I, II and III *Pneumococcus*. Antibodies for the homologous type of *Pneumococcus* appeared at about the time of crisis. Antibodies for heterologous types, on the other hand, appeared usually in the second week of convalescence after 1 to 5 injections of the respective polysaccharides.

A control group of patients, 21 in number, received no skin tests. Their sera, in the great majority of instances, contained antibodies for the homologous type of *Pneumococcus*. In 3 of these cases antibodies for a heterologous type were present but the time of appearance differed from that of the antibodies observed in the first group in that they were detectable in low concentration not only in convalescence but during the acute illness as well. Nor was any increase in concentration observed during convalescence. Antibodies to the heterologous types which were encountered in these 3 cases, apparently preexisted and their appearance is presumably unrelated to any incident occurring in the course of the present infection. The validity of this supposition is borne out by the fact that type-specific antibodies may be found in the serum of certain normal individuals, who give no history of lobar pneumonia. But, in no case were heterologous antibodies first detected in convalescence unless the patient had previously received intradermal injections of the type-specific polysaccharides.

Many investigators have noted the presence of specific antibodies for the homologous strain or type of *Pneumococcus* in the blood of pneumonia patients at the time of crisis. Bacteriotropins (5, 6, 7), agglutinins (5, 7, 8, 9, 10, 11, 12) and protective antibodies (13, 14, 15, 6, 12) have all been demonstrated but so far as can be discovered, in no instance has the development of antibodies for a heterologous type been described. Chickering (11) in 1914 followed the course of agglutinin production to all types throughout convalescence in 40 cases but found only antibodies for the homologous type. Clough (7) in 1919, using the serum of patients convalescent from pneumonia due to known types of *Pneumococcus*, obtained similar results with phagocytic and agglutination reactions. In the sera of 25 normal

individuals, however, Clough (16) found protective antibodies for Type I in 4 of 18 tests, for Type II in 8 of 18 tests; and of 19 tests for Type III antibodies, 8 were positive. Ward (20) tested the capacity of the defibrinated blood of normal individuals to inhibit the growth of pneumococci of Types I, II and III. In each instance a variable degree of pneumococidal action was observed against one or more types of the organism. Furthermore, this property was present in the blood of patients early in the course of pneumonia even against the type of *Pneumococcus* producing the infection. Robertson and Cornwell (21), employing serum-leucocyte mixtures from normal human beings, found that the blood of all persons tested by this method possessed pneumococidal properties for at least one type of *Pneumococcus* and in the majority of instances for two or more types.

In evaluating the factors involved in the production of antibodies for types of *Pneumococcus* heterologous to that causing the infection several possible mechanisms may be considered.

It is possible that antibodies for other types were present before the onset of disease and were masked during the acute infection only to return with convalescence. Or that, present in minimal amounts before the disease, the infection served to cause an outpouring of antibodies in detectable concentrations,—the anamnestic reaction first observed by Cole (17).

Interconvertibility of types as described by Griffith (18) and later studied by Dawson (19) offers a possible explanation. One might assume that with the development of type-specific antibodies to the homologous organism a change of type occurred and, as a result, antibodies to the secondary type appeared.

The evidence presented in this report implicates the polysaccharides employed in intracutaneous tests and suggests that they were antigenic. On the basis of this assumption, the conditions under which the investigation was carried out may have favored antibody production. No previous data are available regarding the antigenicity in humans of the purified type-specific polysaccharides of the *Pneumococcus*. On the basis of animal experiments in this laboratory these substances, in the pure state, are considered non-antigenic. It is possible, however, that in the process of recovery from infection, a highly reactive state exists in the human organism which responds to stimuli otherwise

ineffective. In the removal of the inflammatory material from its focus in the lungs, as takes place in pneumonic resolution, the absorbed material may act as an adjunct to the polysaccharides (the "Schleppe" of the German investigators) forming a combination possessing antigenic properties. The polysaccharides which, under ordinary conditions, are haptens, may then acquire antigenic properties and determine the specificity of the antibodies produced. It is of course also possible that the particular preparations used in this study, although they were prepared with great care, still contained minute amounts of combined polysaccharides.

At present it is impossible to offer any final statement as to the exact mechanism by which antibodies for the heterologous types are produced. Further work is being carried on with normal individuals and patients in an attempt more completely to elucidate the factors which play a rôle in the development of these heterologous antibodies.

SUMMARY

The majority of patients convalescent from pneumonia due to Types I, II and III *Pneumococcus* develop at the time of recovery circulating antibodies for the homologous type of organisms. At the same time an immediate wheal and erythema reaction followed the intradermal injection of the homologous type-specific polysaccharide in 100 per cent of Type I patients, 58.8 per cent of Type II patients, and 44 per cent of Type III patients.

In a group of 18 patients repeatedly tested with the type-specific polysaccharides, 10 developed in the second or third week of convalescence circulating antibodies for one or more heterologous types. In none of 21 control patients was this phenomenon observed.

It is suggested that the development of circulating antibodies for heterologous types of *Pneumococcus* was associated with the previous intradermal injections of the type-specific polysaccharides.

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IMMUNOLOGICAL STUDIES IN RELATION TO THE SUPRARENAL GLAND

VI a. TRYPANOSOMA LEWISI INFECTION IN NORMAL ALBINO RATS VI b. TRYPANOSOMA LEWISI INFECTION IN SUPRARENALECTOMIZED ADULT ALBINO RATS

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VI a. *T. lewisi* Infection in Normal Albino Rats

Though much evidence has been accumulating during recent years to indicate the importance of the reticulo-endothelial cells in resistance and antibody formation, little is known of the more fundamental controlling influences of this mechanism. In previous studies (1-5) we have gathered evidence of the importance of the suprarenal gland in antibody formation through the influence of the water balance in the tissues of the body. That the suprarenal glands are essential in the mechanism of natural resistance has been established through the work of several investigators and in previous work in this laboratory (6-9). In an effort to determine what part the suprarenal gland and other glands of internal secretion play in resistance to protozoan diseases, *T. lewisi* infection, a common disease of rodents, was studied.

T. lewisi infection in the albino rat offers certain advantages for experimental study.* Spontaneous infection is rare, but the disease can be readily transmitted by inoculation of infected blood. Its course can be quantitatively studied. The severity of the infection can be estimated by counts of the trypanosomes in the peripheral blood stream and by estimation of the coefficient of variation in the size of the parasites during the reproductive phase of the infection (12). They produce a mild disease in adult rats and recovery occurs within 4 to 5 weeks, with an acquired immunity that remains permanent.

* For a review of the literature on *T. lewisi* infection, see Laveran and Mesnil (10) and Taliaferro (11).

The wide variation in the course and duration of the infection reported by previous workers (11, 13, 14) may be in part due to the fact that the factors of stock, age, weight of the rats, diet and the environmental conditions of the animals were not uniform. We have kept these factors as constant as possible.

The course of *T. lewisi* infection was studied in 40 normal adult albino rats and was made the basis of comparison with infections in suprarenalectomized, nephrectomized, splenectomized, gonadectomized, and thymectomized rats.

Method

The rats were all of Wistar Institute stock, raised in our laboratory. Except where otherwise specifically stated, they were 3 months of age, and ranged in weight from 150 to 225 gm. They were kept under constant environmental conditions and maintained on a standard adequate diet.

The strain of the *Trypanosoma lewisi* was obtained through the courtesy of Dr. Linton of Columbia University and was maintained by weekly transfers. 1 cc. of blood from rats infected with *T. lewisi* was drawn from the heart and was diluted with 9 parts of physiological salt solution containing 1 per cent sodium citrate. 1 cc. of this dilution was injected intraperitoneally into a series of rats. Red blood cell counts were made at frequent intervals. Smears of the peripheral blood were made daily during the first week and then at 2 day intervals thereafter until the trypanosomes disappeared from the blood stream. Trypanosome counts were estimated and the curve of the infection determined in each instance.

The Course of the Experimental Infection in the Normal Adult Albino Rat

Following intraperitoneal injection the trypanosomes multiplied in the peritoneal cavity for a period of 1 to 3 days. Within 24 to 48 hours trypanosomes appeared in the peripheral blood. They rapidly multiplied and swollen forms became numerous. By the fourth day the count exceeded 100,000 per cubic millimeter. Multiplication forms were present during the first 7 to 9 days and from then on only adult forms were seen. The average interval from the onset to the height of the infection was 7.5 days, the range 5 to 18 days. The number of trypanosomes at the height of infection varied from 115,000 to 800,000 per cubic millimeter. The average number was 336,000 per cubic millimeter. The average duration of the infection was 27.4 days. In two instances the duration of the infection was less than 3

weeks, in sixteen 3 to 4 weeks and in twenty-two 4 to 5 weeks. No instance of infection in our normal rats exceeded 35 days. The average duration of the infection was the same in males and females. In most instances the trypanosomes disappeared from the blood gradually, but in six instances the number of trypanosomes dropped within 24 hours from a high count to zero. In general these findings are consistent with those of previous investigators. The duration of the infection in the rat is not as varied if the factors of stock, age, weight, environment, care and diet are kept uniform.

Symptoms of the Infection.—Laveran and Mesnil (10) observed that very young rats failed to gain weight during the first few days of the infection. A definite clinical syndrome in the adult rat was noted when *T. lewisi* infection was first introduced into our stock. During the first 48 hours, the rat was quiet, his appetite was poor, the eyelids were edematous and sticky, and edema of the penis was present. These symptoms rapidly subsided and the rats appeared normal during the rest of the infection. In two instances during the second week of the infection partial paralysis of the hind limbs was observed which disappeared within 3 weeks. After the first two or three transfers of the strain of *T. lewisi*, symptoms of edema of the lids and penis were no longer noted and no further instance of paralysis was observed. The infection was associated with fewer symptoms when the *Trypanosoma lewisi* strain had undergone several passages.

A moderate anemia developed during the first few days of the infection which was most severe at the height of the infection. The red cell count rarely dropped below 4,500,000. The anemia was associated with the appearance of *Bartonella muris* bodies in the red cells. From experimental studies reported in a previous communication (15) it was found that the virus of *Bartonella muris* anemia could be separated from the trypanosome infected blood by passage through young rabbits. The concomitant anemia was more severe in 3 week old rats than in mature rats and the *T. lewisi* infection ran a more prolonged and severe course in the young rats. Death from trypanosome infection in very young rats may occur with a severe anemia, the red cell count dropping to 1,000,000 or less. *Bartonella muris* bodies are present in large numbers in the red cells. Autopsy of the fatal cases reveals icteric tint to the subcutaneous fat, enlargement of the spleen and fatty changes of the liver, heart and kidneys.

Pathology of the Trypanosoma lewisi Infection in the Normal Adult Rat

A series of 19 rats were inoculated with blood from a rat injected with *Trypanosoma lewisi* and killed at intervals of 2 days from the first day of infection to the 38th day after inoculation. Macroscopically the only pathological changes were noted in the spleen. It enlarged progressively until at the height of the infection it was 6 to 7 times the normal size, amounting to 1.55 per cent of the body weight as compared with 0.27 per cent normally. It diminished in weight after the 10th day but at the end of the infection it was still larger than normal. Histological studies show a progressive enlargement of the follicles and marked congestion of the pulp. The reticular and endothelial elements became engorged with red blood cells, were greatly increased in size and many underwent disintegration. Some of the Kupffer cells of the liver show erythrophagocytosis. In the later stages of the infection the follicles of the spleen diminish in size, the congestion disappears and there is some increase in the connective tissue of the pulp. No trypanosomes were found in the tissues of rats killed at the height of the infection when several hundred thousand trypanosomes per cubic millimeter were present in the peripheral blood stream.

In mice infected with pathogenic trypanosomes the spleen may hypertrophy to 60 times the normal size (16). Taliaferro (11) states that splenic enlargement is more marked in the severe trypanosome infections of mouse, rat, guinea pig and dog,—which die with large numbers of parasites in their blood,—than in the goat, sheep, rabbit and cattle in which trypanosomes are only rarely found.

T. lewisi infection in the rat results in severe injury to the reticular and endothelial elements of the spleen. Evidence of such injury is afforded by the concomitant occurrence in many rats of the *Bartonella* anemia at the height of the trypanosome infection. *T. lewisi* infections produce the same effect as splenectomy. Cannon and McClelland (17) found that repeated injections of large amounts of India ink over a long period of time in normal rats is followed by the appearance of *Bartonella* anemia. As much as 60 to 80 cc. of India ink (4 per cent suspension) was used per rat. They attribute this

anemia to physiological "blockade" of the reticulo-endothelial elements.

Complement Fixation in Trypanosoma lewisi Infection

Although complement fixation in diseases due to the pathogenic trypanosomes has been extensively investigated (11), no studies on complement fixation in *T. lewisi* infection in the rat have been reported to our knowledge.

Manteufel (18) used aqueous and alcoholic extracts of organs of rats infected with *T. lewisi* in *T. equiperdum* studies in horses with contradictory results. Besseman and Leynen (19) in studying methods of obtaining sensitive antigens for use in diagnosis in *T. equiperdum* (dourine) used among other things *T. lewisi* as a source of the test antigen and found it unsatisfactory.

Studies on the complement fixing antibodies of rats recovered from *T. lewisi* were made in an effort to obtain a comparative standard for antibody formation under different conditions. Aqueous and alcoholic antigens were used, prepared from the liver and spleen of rats infected with *T. lewisi*. Saline washings of heated and unheated cultures of *T. lewisi* grown on Novy and McNeal medium were also tested.

Methods.—The saline extracts were made as follows: The spleens of infected rats were macerated and ground in a mortar and shaken with 30 cc. of physiological salt solution per spleen during 30 minutes. The mixture was rapidly filtered and the filtrate preserved in 0.5 per cent phenol. Only freshly prepared extracts were used. The livers of infected rats were macerated with saline and the volume was brought up to 15 cc. per gram of liver. The suspension was shaken thoroughly for 30 minutes, left over night in the ice chest, again shaken and filtered. The anticomplementary titer of the antigens was determined and one-fourth the anticomplementary unit was used in each test. The tests were carried out as follows: 0.1 cc. of inactivated serum, 0.1 cc. of antigen in the dilution estimated by previous titration and 0.1 cc. of guinea pig serum diluted as indicated by previous complement titration ($2\frac{1}{2}$ units). The total volume was brought up to 1 cc. and incubated during 1 hour at 37°. The hemolytic system, consisting of 0.1 cc. of 5 per cent suspension of washed sheep cells and 0.1 cc. of amboceptor in dilution to equal $2\frac{1}{2}$ units was then added.

We have found that during the course of the infection the serum of the infected rat is anticomplementary in very high dilutions, some-

times higher than 1/1000, owing to the fact that both antigen and antibody are present in the serum of the rat during the infection.

Complement fixation in rats recovered from the infection for several weeks were tested with the antigens described. All the antigens gave positive results with positive sera but the strongest reactions were obtained with saline extracts of spleen and liver and saline washings of trypanosome cultures. A sample protocol is given below.

Sera	Saline extract spleen	Saline extract liver	Saline washings trypanosome cultures
Pooled positive*.....	1/400	1/160	1/600
Positive Rat 1.....	0	0	0
Positive Rat 3.....	1/700	1/80	1/1000
Positive Rat 4.....	1/100	1/200	1/400
Positive Rat 5.....	1/200	1/20	1/200
Pooled negative**.....	1/10	1/10	1/10
Negative Rat 2.....	0	0	0

* Positive sera were obtained from rats 2 weeks after they had recovered from *T. lewisi* infection.

** Negative sera were obtained from normal uninfected rats.

Further studies are in progress to determine the course of antibody formation during the life of the rat following infection.

Since the original observations of Rabinowitch and Kempner (20) it is known that a rat which has recovered from an infection with *T. lewisi* cannot be reinfected. The mechanism of this permanent immunity is not understood but studies on antibody formation suggest that the immunity is primarily a humoral one.

Laveran and Mesnil had observed the phagocytosis of *T. lewisi* in the peritoneal cavity of immune rats reinjected with trypanosomes. That phagocytosis by monocytes and circulating leucocytes plays a part in the immune reaction is evident, but many observers have failed to note phagocytosis of trypanosomes.

SUMMARY

T. lewisi infection was studied in a large series of normal adult and young albino rats. The importance of using rats of the same stock, age, and weight for comparative studies with this infection is empha-

sized. The infection produced by intraperitoneal inoculation of blood of an infected rat lasts about 1 month. The height of the infection is reached at about the seventh day. *T. lewisi*, though never fatal in the adult rat, produces a definite disease entity with a progressive splenomegaly during the first 10 days of the disease. Histologically there is hyperplasia of the follicles and congestion of the pulp with marked erythrophagocytosis by reticular and endothelial elements. The spleen diminishes in size with the recovery of the animal. A moderate anemia develops, due to the concomitant infection with *Bartonella muris* viris. During the course of the infection the best index of the resistance of the animal is afforded by the daily trypanosome counts, by the interval during which developmental forms are present and by the duration of the infection. The serum of the rat during this period is highly anticomplementary. After recovery complement fixing antibodies can be detected for a long period of time. The best antigens for the detection of complement fixing antibodies are saline extracts of rat spleens removed at the height of *T. lewisi* infection and saline washings of unkilld cultures of trypanosomes.

VI b. *T. lewisi* Infection in Suprarenalectomized Adult Albino Rats

It has been established that suprarenalectomy in albino rats lowers their resistance to toxins, chemical poisons and bacterial infections and diminishes their capacity for antibody formation (6-9). In an effort to determine the rôle of the suprarenal gland in resistance to protozoan infections, *T. lewisi*, a common protozoan of rats, was studied. In the first part of this communication the course of the infection and its pathology was described. Experiments were next carried out to determine the effect, if any, of suprarenalectomy on the acquired immunity to *T. lewisi* of rats recovered from a first infection.

The experiments were divided into two groups. In the first group eighteen adult rats were suprarenalectomized and in five the suprarenal areas were traumatized. 6 days after the operation all the rats were injected intraperitoneally with 1 cc. of a 10 per cent dilution of whole blood drawn from a rat infected with *T. lewisi*. Smears and counts of the peripheral blood were made at frequent intervals.

The results of this experiment are given in Table I and Fig. 1. It is to be noted that of the 18 rats, 12 or 67 per cent, died within 2 to 19

days. The average duration of life of these rats with a fatal infection was 5.8 days after injection with *T. lewisi*, and the average interval in days between the onset and the height of infection was 4.2 days. The trypanosome count was not greater in the suprarenalectomized rats than in normal rats. Apparently, however, toward the height of the infection, the toxic effect of the trypanosomes was sufficient to kill a large percentage of the suprarenalectomized rats. The average num-

TABLE I

The Effect of Bilateral Suprarenalectomy and Unilateral Nephrectomy on Trypanosoma lewisi Infection

Operation	No. of rats	Days between operation and infect.	Duration of infection in days				Interval in days from onset to height of infection				No. of trypanosomes per cubic mm. at height of infection				Per cent surv.	Per cent died
			Aver.	Min.	Max.	Mean	Aver.	Min.	Max.	Mean	Aver.	Min.	Max.	Mean		
											(Expressed in thousands)					
Bil. supra. fatal instances.....	12	6	5.8	2	19	5	4.2	2	8	4	220	10*	640	212	—	—
Bil. supra. recovered instances.....	6	6	25	12	31	26.5	8.6	5	11	9	338	160	640	305	—	—
Bil. supra. recovered and fatal instances....	18	6	12.5	2	31	6	5.9	2	11	5	229	10*	640	212	33	67
Trauma to supra. area..	5	6	24	16	28	26	6.4	5	8	6	380	166	700	300	100	0
Unil. nephrect.....	8	6	26.7	6	45	32	8	6	10	8	330	100	890	290	100	0
Unoperat. adult normals.	40	—	27.4	6	35	28	7.5	5	18	7	337	113	800	285	100	0

* Animal died 24 hours after infection.

ber of trypanosomes in the suprarenalectomized rats at the height of infection was 338,000 per cubic millimeter as compared with 337,000 for those which had been normal. The average interval between the onset and the height of the infection in those rats which survived the infection was 8.6 days. The average duration of the infection in the

surviving suprarenalectomized rats was 25 days as compared to 27.4 days of the normal group.

During the same period 20 rats were suprarenalectomized as controls. Of these rats, 80 per cent survived more than 1 month. In experiments with several hundred suprarenalectomized rats in this laboratory during the past 5 years it has been found that about 80 per cent of suprarenalectomized rats survive longer than 1 month. The mor-

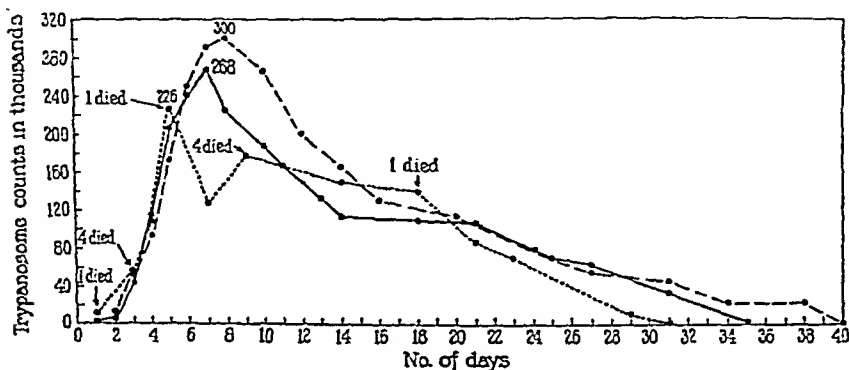


FIG. 1. The effect of bilateral suprarenalectomy, unilateral nephrectomy and traumatization of the suprarenal area on the course of *Trypanosoma lewisi* infection in albino rats.

Each point on the curve of the infection of the suprarenalectomized rats indicates the average count of the surviving animals on that day.

The curves represent the daily average counts expressed in thousands per cubic millimeter.

Normal———

Bilateral suprarenalectomy

Unilateral nephrectomy -----

tality of 70 per cent to *T. lewisi* infection in the suprarenalectomized rats, therefore, indicates a severe drop in resistance.

The curve in Fig. 1 indicates the average daily trypanosome counts. Each point on the chart represents the average count of all the rats living on that day. The curve is practically the same as the curve of the composite average counts of the normal infected group.

An effort was made to reinfect the surviving rats but without success.

Pathology of the T. lewisi Infection in Suprarenalectomized Rats

A comparative study of the pathological changes in the suprarenalectomized group dying of *T. lewisi* infection and the group that had been normal prior to infection did not reveal any striking differences in cellular reaction.

TABLE II

Effect of Trypanosoma lewisi on the Weight of the Spleen in Normal and Suprarenalectomized Rats

Normal adult rats		Adult rats infected with <i>Trypanosoma lewisi</i>			Suprarenalectomized adult rats infected with <i>Trypanosoma lewisi</i>				Suprarenalectomized uninfected adult rats		
No. of rats	13	No.	Days after infection	Per cent weight of spleen	No.	Days after operation	Days after infection	Per cent weight of spleen	No.	Days after operation	Per cent weight of spleen
Per cent weight of spleen to body weight		1001	1	.170	1454	10	4	.687	1458	10	.265
		1002	3	.351	1462*	11	5	.728	1466	11	.288
Aver. wt. .269%		1003	5	.852	1455	12	6	.821	1459	12	.245
Max. wt. .448%		1004	7	1.195	1460	14	8	.645	1461	14	.251
Min. wt. .104%		1005	9	1.316	1463	16	10	.832	1470	19	.224
Mean wt. .268%		1006	11	1.554	1464*	17	11	.707	1471	20	.224
		1007	13	1.248							
		1008	15	.871							
		1009	18	.945							
		1010	20	.900							
		1011	22	.848							
		1012	25	.723							
		1013	28	.900							
		1014	32	1.000							
		1038	36	.670							
		1039	38	.560							

* Died.

Twelve rats were suprarenalectomized and six of these were infected with *T. lewisi* after 6 days. The other six were kept as suprarenalectomized controls. One rat in each group was killed every other day and the pathological changes were noted and compared with those in normal rats infected with *T. lewisi*. The ratio of the spleen weight to the body weight was noted in both groups and compared with the normal. Suprarenalectomy without infection does not cause an increase in the weight of the spleen (Table II), but histological studies made at frequent

intervals reveal a progressive hyperplasia of the follicles with no increase in the total volume. The hyperplasia of the follicles is part of a generalized lymph tissue and thymus hyperplasia evident throughout the body after suprarenalcetomy, as pointed out by Marine (21) and Jaffe (20).

The degree of splenomegaly in the suprarenalcetomized and infected group was definitely less than in the unoperated infected group. On the tenth day after infection the spleen weighed 0.832 per cent of the body weight as compared with 1.554 per cent in the unoperated infected group (Table II). Though the degree of hypertrophy of the spleen is less, the character of the cellular response is the same in both groups. The reticular and endothelial elements are markedly distended with engulfed red blood cells and hemosiderin pigment. The pulp is congested and the follicles prominent.

In the second group of experiments 15 normal adult rats were infected with *T. lewisi* as described above and 1 month after all the trypanosomes had disappeared from the peripheral blood stream, the suprarenal glands were removed. After 5 days all were reinjected with *T. lewisi*. Reinfection did not occur in a single instance. It was not possible to overcome the acquired resistance by suprarenalcetomy, regardless of the quantity of material used for reinjection.

Effect of Unilateral Nephrectomy and Traumatization of the Suprarenal Area on the Course of a Subsequent T. lewisi Infection

In an effort to control the factor of the operative procedure the left kidney was removed from eight adult rats by the posterior route and in five others the tissue about the suprarenal areas was injured. Six days after operation the rats were injected intraperitoneally with *T. lewisi* as described above. From Table I and Fig. 1 it is seen that the infection in these animals did not differ from that of normal rats. The average duration from the onset to the height of infection in the nephrectomized group is 8 days as compared with 7.5 of the normal group. The average number of trypanosomes at the height of infection was 330,000 per cubic millimeter, the range 100,000 to 890,000. All the rats survived the infection. The average duration of infection in the nephrectomized group was 26.7 as compared to 27.4 in the normal. The longest infection lasted 32 days.

One month after recovery all the rats were reinjected with *T. lewisi* but no reinfection was observed.

DISCUSSION

Bilateral suprarenalectomy lowers the resistance of adult albino rats to *T. lewisi* infection. In these rats the disease is fatal in almost 70 per cent of instances whereas of animals previously normal none died. Despite this high mortality the infection as characterized by the rate of growth of the trypanosomes does not differ essentially from the infection in normal rats. Neither the reproduction-inhibiting immune factor (Taliaferro) nor the trypanolytic immune factor is diminished by suprarenalectomy. Apparently the toxic effect of the infection is lethal in these animals. Suprarenalectomy further diminishes the degree of splenic response as estimated by the size of the spleen, but does not alter the reaction of the reticular and endothelial elements of the spleen or of the lymphoid tissue to the *T. lewisi* infection.

One infection with *T. lewisi* confers a permanent immunity in normal rats. It is of significance that suprarenalectomy does not break down the permanent immunity to *T. lewisi* infection acquired as a result of a first infection. This fact emphasizes an essential difference in the mechanisms of natural resistance and acquired resistance. The natural susceptibility of the rat to various toxins and poisons and infections is markedly increased by suprarenalectomy. But the acquired immunity established as a result of a first infection is uninfluenced by suprarenalectomy.

We have found in other studies that the *Bartonella muris* anemia of splenectomized rats cannot be transmitted to suprarenalectomized rats. It has been demonstrated in a previous communication that *Bartonella muris* anemia in the adult splenectomized rat represents a flaring up of a latent infection with the *Bartonella muris* virus and the development of the anemia is indicative of a depression in the acquired immunity of the rat to the virus (15). The failure of the suprarenalectomized rat to develop the anemia either spontaneously or after injection of anemic blood of splenectomized rats indicates that the suprarenal gland does not influence the mechanism of acquired immunity to this infection. This observation with the *Bartonella muris* infection and the fact that the permanent immunity to *T. lewisi*, acquired as a result of a previous infection, is uninfluenced by suprarenalec-

tomy are of significance. Once a humoral or cellular immunity is established to an infection this acquired resistance cannot be sufficiently depressed to permit of a second infection. Acquired resistance and natural resistance are dependent on different physiological processes in the organism and are not merely quantitative variations of the same process as is generally supposed.

SUMMARY AND CONCLUSIONS

Bilateral suprarenalectomy in rats lowers the resistance to a subsequent infection with *T. lewisi*. Almost 70 per cent of these rats die within an average period of 5.8 days after infection. The multiplication of the parasites in the circulating stream is not more considerable than in rats previously normal nor is the duration of the disease in the surviving rats any longer than in the normal group. Bilateral suprarenalectomy does not prevent the formation of immune substances to the parasites but appears to lower the natural resistance of the rat to the toxic effects of the protozoan infection. The acquired immunity to *T. lewisi* of normal rats as a result of infection is not broken down by subsequent suprarenalectomy. Unilateral nephrectomy does not affect the course of a subsequent infection with *T. lewisi*.

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FURTHER STUDIES ON *T. LEWISI* INFECTION IN ALBINO RATS*

I. THE EFFECT OF SPLENECTOMY ON *T. LEWISI* INFECTION IN ALBINO RATS AND THE PROTECTIVE ACTION OF SPLENIC AUTOTRANSPLANTS

II. THE EFFECT OF THYMECTOMY AND BILATERAL GONADECTOMY ON *T. LEWISI* INFECTION IN ALBINO RATS

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The Effect of Splenectomy on the Resistance of Albino Rats to T. lewisi, and the Protective Action of Splenic Autotransplants

In studying the pathology of *T. lewisi* infection in normal rats it was noted that the spleen undergoes marked hyperplasia within a few days after injection of the organism (1). An effort was made to determine the rôle of the spleen in this infection by splenectomy at various intervals prior to infection with *T. lewisi* and to determine the effect of autoplasmic splenic transplants on the infection in splenectomized rats.

The marked involvement of the reticular and endothelial cells of the spleen in the pathogenic trypanosome infections has led to numerous investigations on the effect of splenectomy on the course of these infections. A review of the literature is given by Taliaferro (2).

Bradford and Plimmer (3) found that rats, dogs, cats and rabbits splenectomized before injection with *T. brucei* died earlier than the controls. Laveran and Mesnil (4) observed that splenectomy did not influence the course of *T. nagana* infection in the rat. Rodet and Vallet (5), as well as Sauerbeck (6), believe the spleen possesses striking trypanolytic power. Roux and Lacomme (7) treated three dogs infected with *T. nagana* with splenic extract of normal cattle and found

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that the parasites decreased in number but this may have been due to trypanolytic crises (Rodet and Vallet (5)). In a study on 2 splenectomized rats and 5 guinea pigs, Laveran and Thiroux (8) could find no effect of splenectomy on the course of infection with *T. brucei* and found no trypanolytic action of splenic extracts. They believed the spleen merely removed the débris after trypanolytic crises. Mutermilch (9) found that extracts of spleen produced lysis of *T. brucei* *in vitro*. Bone marrow and liver extracts were also effective but to a less degree, and extracts of other organs had no effect. From this he concluded that the hemato-poietic organs are particularly trypanolytic. For a time it was thought that the spleen acted as a refuge for trypanosomes during the crises, and resistant forms were said to exist. But as Laveran and Mesnil (10) showed, such a theory was unwarranted as isolated trypanosomes could be demonstrated in the blood during the crisis by subinoculation methods. Taliaferro, Johnson and Cannon (2) found that splenectomy had no effect on the course of *T. equinum* infection in mice.

Taliaferro describes the formation of a humoral antibody in *T. lewisi* infections in the rat that inhibits the cell division of the parasite (11) but which does not kill them. Regendanz and Kikuth (12) confirmed these observations. They have further shown that in splenectomized rats division of the trypanosomes continued several days longer than in non-splenectomized controls and in some animals the infection ended fatally. They injected these rats with salvarsan prior to splenectomy to sterilize them of the *Bartonella* virus. Taliaferro, Cannon and Goodloe (13) found little effect if splenectomy was performed before injection of *T. lewisi* but if performed after injection the reproduction of the parasites again occurred, the adult forms beginning to divide. Splenectomy was more effective in the presence of *Bartonella* infection.

The rats of our stock are carriers of the *Bartonella* virus and splenectomy in these rats results in *Bartonella* anemia. We therefore had the opportunity of determining the influence of this infection on *Trypanosoma lewisi* infection.

The experiments were divided into three groups. In the first group 7 rats were infected 6 days after splenectomy at the height of the *Bartonella* anemia. A virulent infection resulted with rapid death of the rats within 3 to 7 days after infection. The average number of trypanosomes at the height of the infection reached 1,200,000 per cubic millimeter, about 4 times greater than the number of trypanosomes in the normal controls. The average duration of life was 11.6 days after injection of the trypanosomes. The height of the infection as estimated by the number of trypanosomes was reached at the time of death (see Table I and Fig. 1). We have found (14) that 100 per cent of our stock develop the *Bartonella*

anemia following splenectomy in from 4 to 10 days. The mortality from the *Bartonella* anemia in 50 splenectomized rats was 20 to 30 per cent. The anemia persists 2 to 3 weeks. The presence of the *Bartonella* infection markedly lowers the resistance of the animal to the *T. lewisi*. The *Bartonella* anemia probably destroys or injures the defensive mechanism (reticulo-endothelial elements?) and permits a more rapid reproduction of the organisms. In a previous communication (14) we have shown that an infection with *T. lewisi* in a rat with intact spleen permits the occurrence of the *Bartonella* anemia,

TABLE I

The Effect of Splenectomy on Trypanosoma lewisi Infection and the Protective Action of Autoplastic Splenic Transplants

Operation	No. of rats	Days between operation and infection	Duration of infection in days				Interval in days from onset to height of infection				No. of trypanosomes per cubic mm. at height of infection				Per cent surv.	Per cent died
			Aver.	Min.	Max.	Mean	Aver.	Min.	Max.	Mean	Aver.	Min.	Max.	Mean		
											(Expressed in thousands)					
Splenectomy**.....	7	6	11.6	3	18	13	11.6	3	18	13	1200	349	2695	1000	0	100
Splenectomy.....	6	48	60	50	68	61	12.6	4	21	12	705	411	1125	600	90	10
Spl. trans.* and splen..	6	28	34.7	28	41	35	9	7	15	7	530	224	787	589	100	0
Unoperated adult normals.....	40	—	27.4	6	35	28	7.5	5	18	7	337	113	800	285	100	0

** These rats died at height of infection.

* Transplants performed 4 weeks prior to splenectomy.

in mild form, as a result of injury to the reticular and endothelial elements of the spleen by this infection.

In the second group of experiments, 6 splenectomized rats were infected with *T. lewisi* 48 days after splenectomy and well after recovery from the *Bartonella* anemia. Of these, one died on the tenth day from the *T. lewisi* infection without a severe anemia. In the other 5 rats, the *T. lewisi* infection was very severe. The average number of the trypanosomes (see Table I) at the height of the infection was 705,000 per cubic millimeter or about twice that of the normal controls. The interval in days from the onset to the height of

the infection which represents the interval during which reproductive forms are present, averaged 12.6 days. Reproductive forms were present several days longer than in the normal rats. The duration of the infection in the rats was twice as long as the normal group, averaging 60 days.

This experiment indicates that the spleen plays an important rôle in the formation of the reproduction inhibiting factor and also the lytic factor in the infection; 48 days after splenectomy the infection with *T. lewisi* is less severe than in the early period. Apparently a compensatory mechanism has been established but this is less effective than is the normal splenic tissue in combating the *T. lewisi* infection.

In the third group of experiments, six rats in which splenic autotransplants had been introduced 4 weeks prior to splenectomy were infected with *T. lewisi* 28 days after splenectomy and 8 weeks after transplants had been made. The method of transplantation has been described (15). The transplants at the time the spleen was removed were insufficiently regenerated to prevent the occurrence of *Bartonella* anemia. The results of this experiment are indicated in Table I. The average number of trypanosomes at the height of the infection was 530,000 per cubic millimeter or midway between the splenectomized group infected 48 days after splenectomy and the normal group. The interval from the onset to the height of the infection was 9 days in this group as compared to 7.4 days of the normal rats and 12.6 days of the splenectomized rats without transplants. The duration of the infection in these rats was practically the same as in the normal group. The transplants reduced the severity of the infection of splenectomized rats to *T. lewisi*. Though they did not protect the rat against *Bartonella* anemia 4 weeks after they were implanted, the transplants at the end of 8 weeks, or 4 weeks after splenectomy, had regenerated sufficiently to raise the resistance of the animal to *T. lewisi*. Histological examination of these transplants (15) revealed complete regeneration of all elements of the spleen. They appeared as small spleens in the abdominal wall.

Reinfection in Splenectomized Rats.—An attempt was made to determine the rôle of the spleen in the acquired immunity of the rat to reinfection with *T. lewisi*. Ten normal rats were infected with *T. lewisi*. One month after recovery the spleens were removed from these rats.

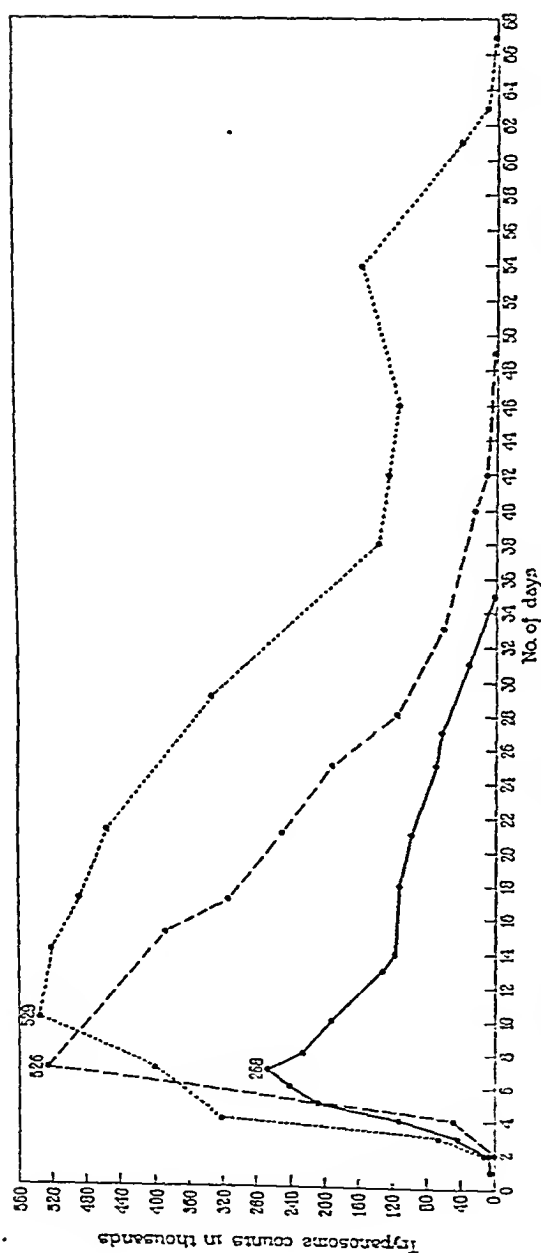


FIG. 1. The effect of splenectomy, and of splenectomy in rats with autotransplants of splenic tissue, on the course of *T. lewisi* infection in adult albino rats.

The curves represent the daily average counts expressed in thousands per cubic millimeter.

Normal —

Splenectomized rats infected 48 days after operation

Splenectomized rats with splenic autotransplants performed 4 weeks prior to splenectomy and infected 24 days after splenectomy — — — — —

Six days later all were reinjected with *T. lewisi*. In only one instance did they persist in the blood for a period of 48 hours. No developmental forms appeared. Repeated injections of *T. lewisi* were made but no reinfection occurred.

DISCUSSION

Our observations are consistent with the findings of Regendanz and Kikuth (12), who studied the effect of splenectomy on the course of *Trypanosoma lewisi* infection in rats. Prior to splenectomy, the rats were injected with salvarsan to free them from the *Bartonella* virus. They found that the infection in the splenectomized rats was more severe than in the normal rats. They did not, however, make quantitative estimates of the course of the infection. The experiments reported in our communication indicate that the spleen plays a very important rôle in the defense mechanism to *T. lewisi*. The capacity of the rat to produce both the immune substance which inhibits the reproduction of the trypanosomes (reproduction-inhibiting immune factor) and the trypanolytic immune substance are markedly reduced by the removal of the spleen, even though the rats are not infected with *T. lewisi* until 7 weeks after the operation. This supports the contention of Taliaferro (2) that the reticulo-endothelial elements of the spleen are probably the main source of these immune substances.

The fact, however, that removal of the spleen does not influence the acquired immunity to *T. lewisi* following a first infection suggests that the immune defense mechanism may be a humoral one. In *Bartonella* infection on the other hand immunity may be a cellular one, dependent on the reticular and the endothelial elements of the spleen. In this disease an acquired immunity is broken down by splenectomy, but not by other procedures such as thymectomy, suprarenalectomy or gonadectomy. In trypanosomiasis the acquired immunity is a humoral one and hence is difficult to destroy. Complement fixing antibodies are present in the blood of rats for months after recovery from *T. lewisi* but no protective immune substance has thus far been demonstrated in the blood of rats either during the infection with *Bartonella* anemia or following it (Ford).

The conflicting results of investigations on the effect of splenectomy

on the pathogenic trypanosome infections is due in part to the difference in the species of experimental animal used. The spleen of the rat or mouse is considerably larger in proportion to the body weight than that of the rabbit or guinea pig. Taliaferro (2) suggests that the quantity of the reticular and endothelial cells in the spleen of the rabbit and guinea pig represents only a small part of the reticulo-endothelial tissue of the body. He believes that splenectomy in these animals therefore has little effect on the course of the trypanosome infections. This assumption however is based on insufficient evidence. It is possible that the relative quantity of hemolymph tissue in these different animals may bear some relation to the severity of the effects of splenectomy. Though it is still a debated question the true hemolymph nodes can probably be considered as accessory splenic tissue. The quantity of this tissue in the rat is extremely small. Macmillan (16) was able to find only 4 hemolymph nodes in the rat, 2 just above the kidney near the midline and 2 at the apex of the lungs in the posterior mediastinum. The rabbit and guinea pig, the sheep and the goat have relatively much more hemolymph tissue. The domestic pig on the other hand is said to have no true hemolymph nodes as determined by lymphatic injection, though many "red" nodes (Meyer (17)). Warthin (18) has demonstrated in the sheep and the goat a marked hypertrophy of the hemolymph nodes within the first few weeks following splenectomy, and considers these nodes as accessory erythropoietic tissue. It is possible that the hemolymph nodes in splenectomized animals assume a specific protective action which is normally a function of splenic tissue.

The Effect of Thymectomy in Young and Adult Rats on T. lewisi Infection

The effect of thymectomy on the course of *T. lewisi* infection in young and adult rats was studied. The rats were divided into two groups. In the first group of experiments 20 6-week old albino rats were used. Ten of these were thymectomized and 10 were kept as normal controls. In the second group, 10 adult, 3-month old rats were thymectomized. One week after operation, the rats of both groups were infected with *T. lewisi*.

Method.—Ether anaesthesia was used. The skin over the sternum was incised vertically and retracted. A purse string suture was put through the pectoral muscles, laterally and superiorly through the loose areolar tissue of the neck and through the submaxillary gland on one side. A similar purse string suture was put through the skin. The sternum was cut with small dull pointed scissors in the midline from above downward for a distance of 1.5 cm., care being taken to cut exactly in the midline to avoid the great vessels. The thymus was exposed, rapidly removed with fine mouse tooth forceps from below upward, and the purse string sutures were rapidly tied. If the chest is allowed to remain open longer than a few seconds the animal may succumb. If the rat survives the operation, it makes a rapid recovery.

The results of this experiment are tabulated in Table II and in the curves of the average daily counts of the trypanosomes in the circulating blood (Fig. 2). The course of the infection in 6-week old rats was slightly different from that of normal rats. The average number of trypanosomes at the height of infection was 316,000 per cubic millimeter as compared with 337,000 in the adult normal rats (see Paper VI, same issue of the journal (1)). The height of the infection was reached, however, somewhat earlier. In the adult normals the interval between the onset and the height of infection is 7.5 days, whereas in the younger group the interval between the onset and the height of infection is 6.4 days. The duration of the infection in the young rats is somewhat less than in the adult rats—22 days average duration in the young as compared to 27.4 days in the adult group. Thymectomy in the young rat has a decidedly beneficial effect on the course of the infection. The average number of trypanosomes at the height of infection is 176,500 per cubic millimeter and the interval between onset and the height of infection is 5 days. In the curve of the daily average counts the height of infection is reached on the 4th day and the average count on this day is 137,000. In the curve of the daily counts of the control young rats, the height of infection is on the 7th day and the average count on this day is double that of the thymectomy group, 273,000. The duration of the infection of the thymectomized rats is decidedly shorter than in the normal young controls. The average duration in the young controls is 22 days, and in the thymectomized young rats, 16.6 days.

The course and duration of the *T. lewisi* infection in adult thymectomized rats was likewise somewhat shorter than in adult normal rats.

The average number of trypanosomes at the height of infection was 241,000 and the interval between the onset and the height was 6.6 days. In the controls the average number of trypanosomes at the height of the infection was 337,000 and the interval from the onset to the height of infection was 7.5 days. The duration of the infection in the thymectomized adult rats is 19.1 days as compared with an average of 27.4 days in the control group.

Thymectomy has a favorable effect on the course of a subsequent infection with the *Trypanosoma lewisi* infection in both young and adult rats, but especially in young rats. The duration of the infection is decidedly less and the intensity of the infection is diminished. Developmental forms are observed during a shorter period and abortive infections are more frequently observed. The removal of the thymus, particularly in the young, has a stimulating effect on the formation of immune substances that inhibit the reproduction of the trypanosomes and have a stimulating effect on the formation of trypanolytic substances of the serum.

Effect of Unilateral and Bilateral Gonadectomy on T. lewisi Infection

Unilateral gonadectomy was performed on six adult albino rats and bilateral gonadectomy on six rats. One week after operation the rats were infected with *T. lewisi*.

Method.—Under ether, a median incision was made in the abdominal wall. The testes were gently drawn into the abdominal cavity by gentle traction on the spermatic cord. The pedicle was ligated and the testes were removed. Care was taken to avoid unnecessary manipulation of the spermatic cords. The abdominal wound was sutured. The rats recovered rapidly from the operation.

The results of this experiment are given in Table II and in Fig. 2. Unilateral gonadectomy in the adult rat has no effect on the trypanosome infection. The average number of trypanosomes at the height of infection is 276,000, reached in an average interval of 6.6 days as compared with the average number of trypanosomes at the height of the infection 337,000 in an average interval of 7.4 days. In the curve of the daily average counts the height is reached on the 7th day with 236,000 per cubic millimeter as compared with the normal 268,000 on the 7th day. Unilateral gonadectomy does not influence the course of a subsequent *Trypanosoma lewisi* infection.

TABLE II

Effect of Thymectomy and Gonadectomy on Trypanosoma lewisi Infection

Operation	No. of rats	Days between operation and infection	Duration of infection in days				Interval in days from onset to height of infection				No. of trypanosomes per cubic mm. at height of infection				Per cent surv.	Per cent died
			Aver.	Min.	Max.	Mean	Aver.	Min.	Max.	Mean	Aver.	Min.	Max.	Mean		
											(Expressed in thousands)					
Thymect. in adults.....	10	6	19.1	7	27	22	6.6	4	8	8	241	60	570	196	100	0
6 week thymect.....	10	—	16.5	6	24	20	5	4	6	5	176	60	315	160	100	0
Unilat. gonadect.....	6	6	28	17	32	27.5	6.6	5	9	6	276	140	428	290	100	0
Bilat. gonadect.....	6	6	26.5	25	31	25.5	7.2	7	9	7	889	550	1186	930	100	0
Unoperated adult normals.....	40	—	27.4	6	35	28	7.5	5	18	7	337	113	800	285	100	0
Unoperated 6 week normals.....	10	—	22	17	27	22	6.4	4	8	7	316	60	570	225	100	0

TABLE III

Operation	No. of rats	Days between operation and infection	Duration of infection in days				Interval in days from onset to height of infection				No. of trypanosomes per cubic mm. at height of infection				Per cent surv.	Per cent died
			Aver.	Min.	Max.	Mean	Aver.	Min.	Max.	Mean	Aver.	Min.	Max.	Mean		
											(Expressed in thousands)					
Bilat.*** supraren.....	12	6	5.8	2	19	5	4.2	2	8	4	220	10	640	212	—	—
Bilat. supraren.....	6	6	25	12	31	26.5	8.6	5	11	9	338	160	640	305	33	67
Splenectomy**.....	7	6	11.6	3	18	13	11.6	3	18	13	1200	349	2695	1000	0	100
Splenectomy.....	6	48	60	50	68	61	12.6	4	21	12	705	411	1125	600	90	10
Spl. trans.* and splen.....	6	28	34.7	28	41	35	9	7	15	7	530	224	787	589	100	0
Unil. nephrect.....	8	6	26.7	6	45	32	8	6	10	8	330	100	890	290	100	0
Thymect. in adults....	10	6	19.1	7	27	22	6.6	4	8	8	241	60	570	196	100	0
6 week thymect.....	10	6	16.5	6	24	20	5	4	6	5	176	60	315	160	100	0
Unil. gonadect.....	6	6	28	17	32	27.5	6.6	5	9	6	276	140	428	290	100	0
Bilat. gonadect.....	6	6	26.5	25	31	25.5	7.2	7	9	7	889	550	1186	930	100	0
Trauma to supr. area.	5	6	24	16	28	25	6.4	5	8	6	380	166	700	300	100	0
Unoperated adult normals.....	40	—	27.4	6	35	28	7.5	5	18	7	337	113	800	285	100	0
Unoperated 6 week normals.....	10	—	22	17	27	22	6.4	4	8	7	316	60	570	225	100	0

*** Died during infection.

** Died at height of infection.

* Transplants performed 4 weeks prior to splenectomy.

Bilateral gonadectomy decidedly depresses the resistance to *T. lewisi*. Though the average duration of the infection is essentially

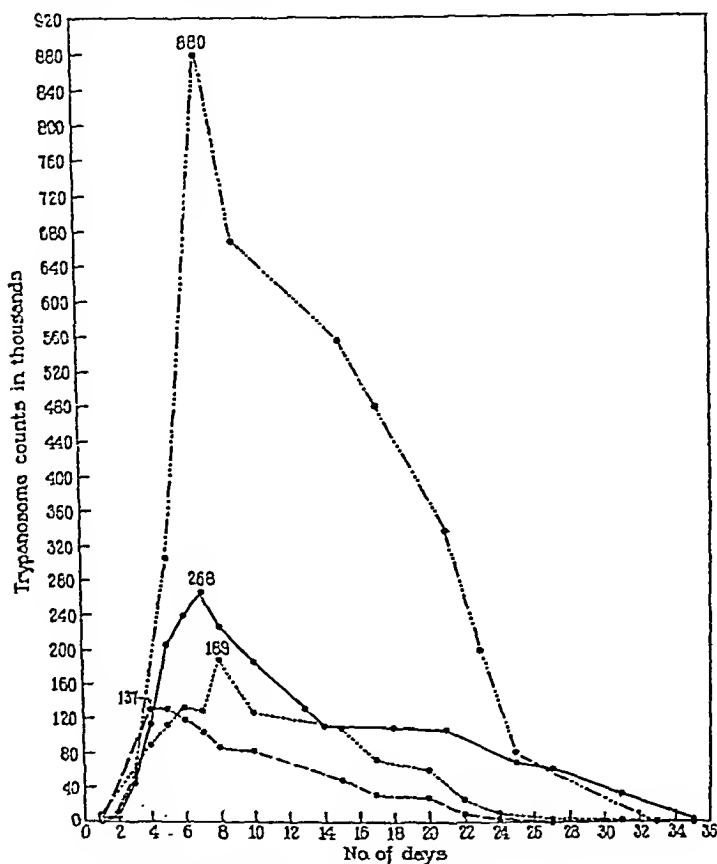


FIG. 2. The effect of bilateral gonadectomy and of thymectomy in young and adult albino rats on the course of *T. lewisi* infection.

The curves represent the daily average counts expressed in thousands per cubic millimeter.

Normal —————

Thymectomy in adult rat

Thymectomy in young rats ————

Bilateral gonadectomy

the same as in the normal group, 26.5 days, the severity of the infection is markedly increased. The average number of trypanosomes

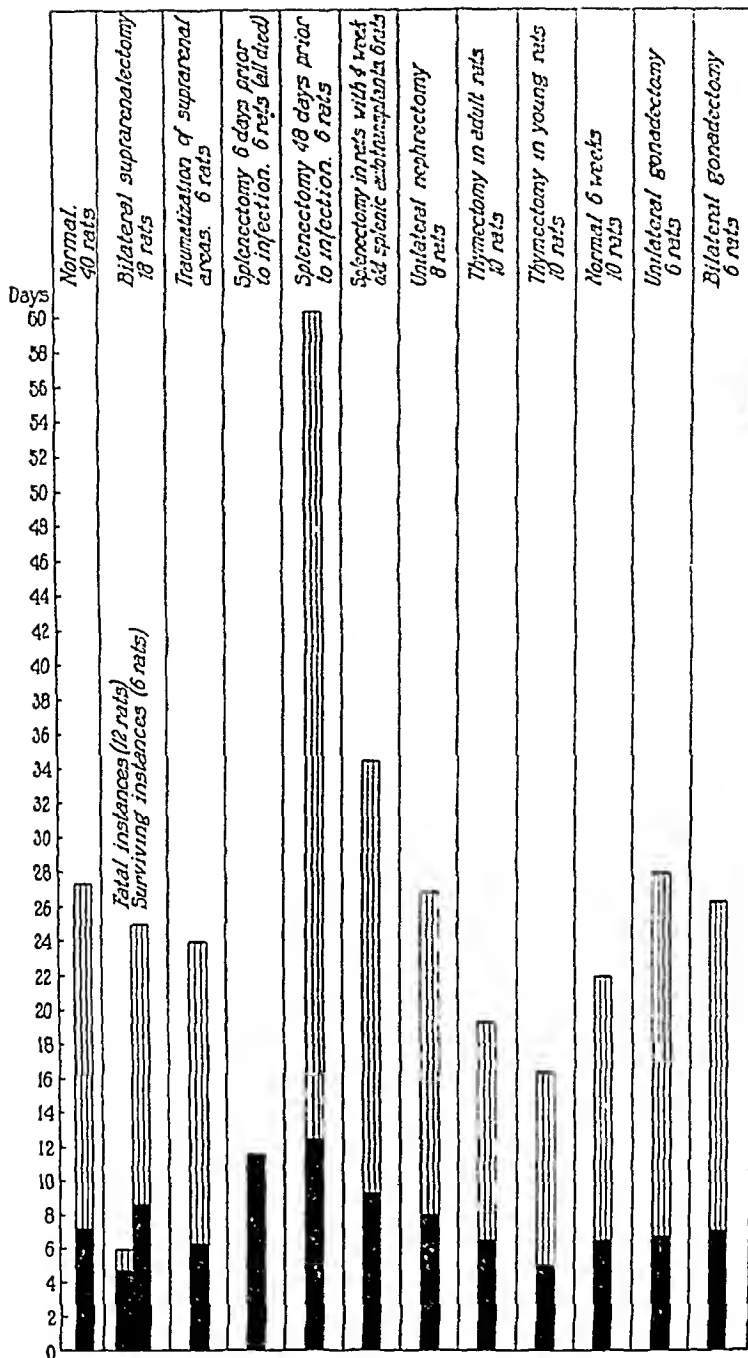


FIG. 3. The duration of infection with *T. lewisi* in normal rats, rats with bilateral suprarenalectomy, traumatization of the suprarenal area, unilateral nephrectomy, unilateral gonadectomy, bilateral gonadectomy, young and adult rats with thymectomy, rats in which splenectomy was performed 6 days prior to infection, rats in which splenectomy was performed 48 days prior to infection, rats with splenectomy in which splenic autotransplants were performed 4 weeks prior to splenectomy.

The solid portion of the bars represents the interval in days from the onset of the infection to the height of the infection.

The entire column represents the average duration in days of the infection.

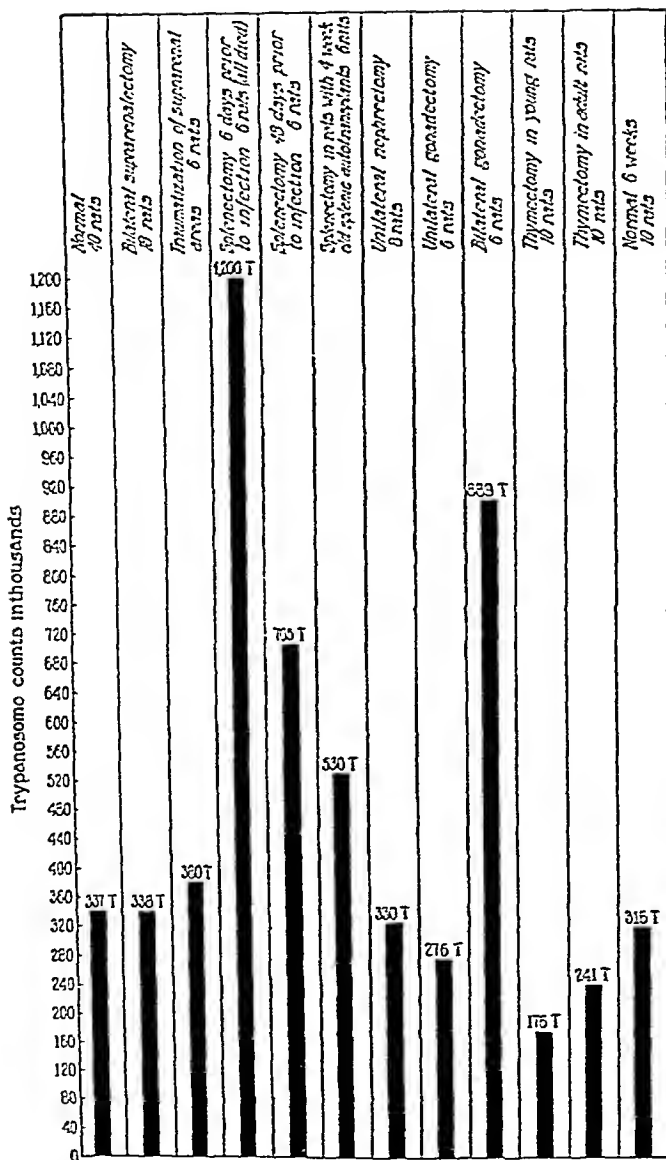


FIG. 4. The number of trypanosomes at the height of infection in rats with bilateral suprarenalectomy, transection of suprarenal area, unilateral gonadectomy and bilateral gonadectomy, thymectomy in young rats, thymectomy in adult rats, splenectomy in rats infected 6 days after operation, splenectomy in rats infected 48 days after operation, splenectomy in rats in which splenic autotransplants had been performed 4 weeks prior to splenectomy.

The bars represent the average number of trypanosomes at the height of infection with *T. lewisi*.

at the height of infection is 889,000 or almost three times as many as the normal group. The curve of the daily average counts reached a peak of 880,000 on the 7th day as compared with 268,000 on the 7th day in the normals. In 3 of the rats with bilateral gonadectomy the count exceeded 1,000,000 per cubic millimeter. Splenectomy resulted in similarly high counts. However, the disease in the bilaterally gonadectomized rats did not last longer than in the normals nor were there any fatalities. We must conclude that removal of the gonads interferes with the formation of the immune substance which inhibits reproduction of the trypanosomes, but does not interfere with the formation of the trypanolytic antibodies. In 4 instances in this group the disease terminated sharply by crisis, which is less frequently the case in normal rats.

Thymectomy in young rats diminishes the severity of the infection and shortens its course. Bilateral gonadectomy in the adult increases the severity of the infection but does not influence the duration of the infection.

SUMMARY

T. lewisi infection in normal adult 3 month old albino rats raised from a single stock and maintained under identical conditions was studied. Daily quantitative estimates of the trypanosomes in the circulating blood were made and the course of the infection was studied. Bilateral suprarenalectomy in rats lowers the resistance to a subsequent infection with *T. lewisi*. About 70 per cent of these rats die in an average period of 5.8 days after injection. The multiplication of the parasites, in the circulating stream, however, is not more considerable in the suprarenalectomized than in the previously normal rats, nor is the duration of the disease in the surviving rats any longer than in the normal group. The removal of the suprarenal glands does not alter the immune reaction to the parasite, but lowers the natural resistance of the animal to the toxic effects of the protozoan infection. Bilateral suprarenalectomy does not lessen the immunity of rats recovered from *T. lewisi* infection to subsequent infection. Unilateral nephrectomy does not influence the course of a subsequent infection with *T. lewisi* infection. The mortality of splenectomized rats from *Bartonella muris* anemia increases from 30 to 100 per cent following

the injection of *T. lewisi* at the height of the anemia 7 days after splenectomy. *T. lewisi* infection 48 days after splenectomy that is to say at a time when the *Bartonella* anemia is no longer present produces a more severe infection than in normal rats. The number of trypanosomes at the height of infection averages 3 times the ordinary and the infection endures twice as long. Both the immune substance that inhibits the reproduction of the parasite and the lytic factor are markedly depressed. Splenic autotransplantation performed 4 weeks prior to splenectomy raises the resistance of rats to a subsequent *T. lewisi* infection. Thymectomy in 6 week old rats diminishes the severity of a subsequent trypanosome infection and shortens its course. Both the formation of the immune substance which inhibits reproduction of the trypanosomes and formation of trypanolytic antibodies are stimulated by this procedure. In the adult rat thymectomy shortens the course of the infection but the severity is only slightly diminished. Bilateral gonadectomy in the adult increases the severity of the infection. The number of trypanosomes at the height of the infection is almost three times the normal. However, the duration of the infection is the same as in the normal rats. The reproduction-inhibiting factor is depressed by bilateral gonadectomy but not the trypanocidal factor. Unilateral gonadectomy does not influence the infection.

We wish to thank Dr. David Marine for his helpful criticism throughout the course of this work.

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BACTERIUM GRANULOSIS IN RELATION TO TRACHOMA: ITS RECOVERY FROM EXPERIMENTALLY INFECTED MONKEYS AND FROM HUMAN TRACHOMA

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PLATE 26

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Since Noguchi's report in 1928 (1), reports have come from laboratories in various parts of the world (Stepanowa and Azarowa in Kharkov (2), Finnoff and Thygeson in Denver (3), Addario in Palermo (4), Kendall in Chicago (5), Tilden and Tyler (6) and Olitsky and Tyler (7) in New York, and Lumbroso in Tunis (8)) of the isolation of *Bacterium granulosis* from trachoma and the reproduction in monkeys of granulomatous lesions of the conjunctiva such as were originally secured by Noguchi. It has now become desirable to assemble as many strains as possible of both European and American origin, in order that a comparative study can be carried out. Since the cultures survive for many weeks, even months, on the semisolid leptospira medium at ordinary temperatures, when sealed in ampoules, such a study is entirely feasible.

Certain animals described in Noguchi's monograph are still alive, $2\frac{1}{2}$ years after inoculation, and continue to show the granulomatous lesions. The lesions in the chimpanzee "Louisa," which reached their maximum development within a period of 8 months, have since remained stationary. Of the *Macacus rhesus*, No. 80 shows, at the end of $2\frac{1}{2}$ years, complete healing, with scar tissue, in the left eye (Fig. 2), while the right lid still carries a few follicles. Two *Macacus rhesus* (Nos. 5 and 6) inoculated from *Macacus* 73 in February, 1928, continue to have pronounced lesions. In June, 1929, No. 5 showed a spontaneous exacerbation, with marked redness and lacrimation, the cornea becoming diffusely cloudy (Fig. 3).

The present report deals with the following experiments: (1) the further transmission (a) by animal passage, and (b) by inoculation of cultures, of the Albuquerque Strain 1, isolated by Noguchi from Indian trachoma in 1926; (2) the recovery of the organism from monkey lesions, in their early stages, and also after they had persisted for a year or longer; (3) the isolation of new strains from cases of Indian trachoma, and (4) comparative cultural studies of the old and new strains and viability tests of the bacterium. The isolation of new strains was made possible through the cooperation of Dr. F. I. Proctor and the U. S. Indian Service. For clinical assistance we are indebted to Drs. Polk Richards and J. F. Lane, of the Indian Service.

1. Experiments with Albuquerque Strain 1 (Noguchi)

(a) Transmission by Animal Passage.—

The transfer of the infection from monkey to monkey was accomplished by means of tissue suspensions, the entire affected tarsal conjunctiva being removed, under ether anesthesia, ground in a mortar with a minimum quantity of isotonic sodium chloride solution, and injected subconjunctivally and also applied to the scarified conjunctiva, as described by Noguchi.

The first passage from two monkeys injected with cultures by Noguchi in June, 1927, (Nos. 72 and 73) gave positive results in most of the animals inoculated (Table 1). The only exception was an experiment (9) in which the suspension from the right conjunctiva of No. 72 was distributed among 15 animals. This diluted material failed in all but one instance to produce infection. With later passages from the same series the incubation period became longer and the positive results less frequent, and further transmissions were abandoned in favor of experiments with cultures.

*(b) Transmission by Means of Cultures.—*Although the original cultures had been carried on artificial medium for a period of $2\frac{1}{2}$ to 3 years, having been transplanted every 6 to 8 weeks, inoculation tests gave positive results (Fig. 4) in about one-third of the animals (Table 2), showing that *Bacterium granulosis* may retain its pathogenicity for long periods of artificial cultivation.

TABLE 1
Transmission of Albuquerque Strain 1 by Passage

Monkey No.	Inoculated		Incubation period	Result		Remarks
	Date	Material		Left*	Right	
	1927		days			
1	Dec. 9	Conj. susp. No. 72 N**	13	++++	++++	Lesions persisted 1½ years. Died
2	"	"	13	++++	+	Lesions persisted 1 year. Right eye negative until 11 months after inoculation
	1928					
3 ¹	Feb. 10	Conj. susp. No. 73 N**		—	—	
4 ¹	"	"		—	—	
5 ¹	"	"	66	++++	++++	Lesions still present (2 years). Cultures recovered
6 ¹	"	"	45	++	++	Receded in 1 month
7 ²	"	"		—	—	
8 ¹	"	"	45	++++	++++	Lesions on right still present. Cultures recovered
9 ²	"	"	33	++++	++++	Lesions persisted 20 months. Cultures negative. Died
10 ²	"	"		—	—	
11	"	"	66	+++	++++	Lesions still present after 19 months. Cultures recovered. Discarded
12	"	"	33	++++	++++	Lesions persisted 16 months. Discarded
13	"	"	139	+++	+++	Lesions persisted 8 months. Discarded

* Inoculations are always made into the left conjunctiva under novocain anesthesia (2 per cent).

** Numbers followed by N refer to monkeys inoculated by Noguchi, and protocols will be found in his report.

¹ Received 5 subcutaneous injections of heat-killed cultures of Strain 1 Noguchi on Jan. 9, 13, 17, 24, and 31.

² Received 5 intravenous injections of cultures of Strain 1 Noguchi on Jan. 9, 13, 17, 24, and 31.

TABLE 1—*Concluded*

Monkey No.	Inoculated		Incubation period	Result		Remarks
	Date	Material		Left*	Right	
	1928		days			
14	Feb. 10	Conj. susp. No. 73 N**	66	+++	+++	Lesions persisted 14 months. Discarded
15	"	"		—	—	
16	Mar. 9	Conj. susp. No. 72 N		—	—	Reinoculated May 16, 1928
17	"	"		—	—	Died of tuberculosis May 16, 1928
18	"	"	24	++	+	Lesions persisted 15 months, but did not progress further
19	Apr. 13	Conj. susp. No. 2		—	—	
20	"	"		—	—	
21	"	"		—	—	
22	"	"		—	—	
16	May 16	Conj. susp. Nos. 86 N, 79 N, and No. 1	83	—	++++	Died of tuberculosis in 3 months
23	"	"		—	—	
24	"	"		—	—	
25	"	"		—	—	
26	"	"		—	—	
"June" chim-panzee	June 20	Conj. susp. "Venus" and 81 N	66	+++	++++	Lesions persisted 1 year; disappearing
31	"	"	48	—	++	Died in 4 months
32	"	"	132	+++	—	Lesions persisted 13 months and remained unilateral. Discarded
33	"	"		—	—	
34	"	"		—	—	

TABLE 2

Inoculations with Routine Subcultures of Bacterium granulosis (Strain 1 Noguchi)

Monkey No.	Inoculated		Incubation period	Result		Remarks
	Date	Material		Left	Right	
27	1928 June 12	Cultures on horse blood and horse serum agar plus carbohydrates and on semi-solid medium, 4 and 13 days old	16 days	++++	++++	Lesions persisted 6 months. Cultures negative at 176 days, transmission positive
28	"	"		-	-	Discarded after 140 days
29	"	"		-	-	"
30	"	"		-	-	"
"Paulina" chimpanzee	Aug. 8	Similar cultures, 4 days old	16 days	+	+	Few follicles at border of tarsus. Did not progress further
35	"	"		-	-	
36	"	"		-	-	
37	"	"		-	-	
38	"	"		-	-	
"Julia" chimpanzee	Nov. 21	Similar cultures, 4 and 15 days old	42 days	++	++	Few granules at border of tarsus, and on tarsal plate. Did not progress further
41	"	"		-	-	Discarded after 6 months
42	"	"		-	-	Discarded after 7 months
43	"	"	5 mos.	++	++	Lesions receded after 2 months
44	"	"		-	-	Discarded after 117 days
61	1929 Jan. 24	Similar cultures, 6 days old	11 days	++++	++++	Lesions progressed for 8 months. Cultures positive at 4 months. Few follicles remaining after 11 months. Discarded
62	"	"	11 days	++	++	Transient lesions, disappeared in 4 months
63	"	"	11 days	+++	+++	Died in 3 months

2. Recovery of *Bacterium granulosis* (Albuquerque Strain 1) from the Monkey Lesions

The material used for isolation experiments was similar to that employed for direct passage. The basic medium was 2 per cent nutrient agar, adjusted to pH

TABLE 3
Recovery of Bacterium granulosis from Monkey Lesions

Monkey No.	Inoculated		Incubation days	Result		Date cultured	Result
	Date	Material		Left	Right		
81 N	1927 Oct. 13	Conj. susp. "Louisa"	89	++++	++++	Dec. 5, 1928 (390 days)	+
5	1928 Feb. 10	Conj. susp. 73 N	66	++++	++++	Dec. 5, 1928 (299 days)	+
27	June 12	Strain 1 cultures	16	++++	++	Dec. 5, 1928 (176 days)	-
8	Feb. 10	Conj. susp. 73 N	45	++++	++++	Jan. 31, 1929 (356 days)	+
12	"	"	33	+++	+++	Jan. 31, 1929 (356 days)	-
46	Dec. 5	Conj. susp. No. 5	47	+++	+++	Jan. 31, 1929 (57 days)	+
9	Feb. 10	Conj. susp. No. 73 N	33	++++	++++	Mar. 20, 1929 (405 days)	-
11	"	"	45	++++	+++	Mar. 20, 1929 (405 days)	+
18	Mar. 9	Conj. susp. No. 72 N	24	++	+	Mar. 20, 1929 (377 days)	-
61	1929 Jan. 24	Strain 1 cultures	11	++++	++++	Apr. 5, 1929 (71 days)	+
Chimpan- zee "Louisa"	1927 May 20	Conj. susp. chim- panzee "Kitty" (3d passage)	28	++++	++++	May 8, 1930 (1083 days)	+

7.4, to each 100 cc. of which was added 12 cc. of defibrinated horse blood and 5 cc. of a Berkefeld V filtrate of a mixture of 10 per cent dextrose, 10 per cent saccharose, 2.5 per cent mannose, 2.5 per cent levulose, and 2.5 per cent inulin. The conjunctival suspensions were smeared over the freshly prepared plates, which were then strapped with adhesive tape and incubated at 28°C. for 3 to 6 days.

TABLE 4
Inoculation of Cultures Recovered from Monkeys

Monkey No.	Inoculated		Incubation period	Result		Remarks
	Date	Material		Left	Right	
	1928					
55	Dec. 12	No. 81 N cultures, I gen.,	147 days	—	—	Discarded after 177 days
56	"	52 hrs. old, semisolid medium		++++	++++	Lesions persisted 9 months
57	"			—	—	
58	"		100 days	—	—	
51	"	No. 5 cultures, I gen. 52 hrs. old, semisolid medium		++++	++++	Marked congestion and secretion noted 65th day of disease (Fig. 5). Cultures (secretion) negative
52	"	"		—	—	
53	"	"		—	—	
54	"	"		—	—	
	1929					
60	Jan. 16	No. 5 cultures, 12 days old, blood agar and semi-solid	142 days	++++	++++	Lesions persisted 4 months. Still marked when animal was discarded
59	"	"		—	—	
64	Jan. 24	No. 5 cultures, 6 days old, blood agar and semi-solid	11 days	+++	+++	Lesions progressed for 6 months
65	"	"	11 days	+++	+++	"
66	"	"	11 days	+++	+++	"
67	"	No. 81 N cultures 6 days, blood agar and semisolid	22 days	+++	+++	Lesions persisted 6 months
68	"	"	11 days	+	+	Died of pneumonia, 4 months
69	"	"	11 days	+++	+++	Receding after 7 months. Discarded
45	Apr. 3	No. 8 cultures I, II, III gen.		—	—	
47	"	"	35 days	+	±	Negative after 5 months
48	"	"		—	—	

TABLE 4—*Concluded*

Monkey No.	Inoculated		Incubation period	Result		Remarks
	Date	Material		Left	Right	
52	1929 Apr. 3	No. 46 cultures I, II, III gen.	35 days	++++	++++	Lesions persisted 7 months
53	"	"		—	—	
54	"	"	65 days	++++	++++	Lesions persisted 7 months
70	"	No. 61 cultures III gen., blood agar and semi-solid	1-2 mos.	++	—	Transient lesions
71	"	"	21 days	++++	++++	Lesions persisted 6 months. Discarded
72	"	"		—	—	
73	"	"	21 days	++++	++++	Lesions persisting (10 months)
74	May 17	No. 11 cultures blood agar and semisolid IV gen.		—	—	Discarded after 4 months
75	"	"		—	—	"
76	"	"		—	—	"
77	"	"		—	—	"

Cultures were recovered from 6 of 10 *Macacus rhesus* cultured, and from the chimpanzee "Louisa." With one exception, these animals had all been infected by passage from other monkeys; one *rhesus* had received cultures. The periods elapsing between inoculation and culture varied from 57 days to nearly 3 years (1083 days), and the length of this period seems to have had no relation to the success of recovery. The recovery of the organism from the chimpanzee "Louisa" 3 years after inoculation shows that the organism may persist in the experimental infection, as in the human disease, for a long period of time.

The cultures recovered in some instances proved highly virulent (Fig. 5).

3. Recovery of *Bacterium granulosis* from Human Trachoma Lesions

(a) *Fort Defiance Cases.*—In May, 1929, an expedition was made to Fort Defiance, Arizona, where is situated one of the two special schools

which the United States Government maintains for the segregation of Indian children having trachoma. Five children who had been receiving daily treatment with copper sulfate or silver nitrate and two with advanced trachoma who had never received any treatment were selected for study.

Case 1. K., female, probably between 7 and 9 years old. Has been in the school 1 year; had advanced trachoma when she came. Scar tissue present. Treated with silver nitrate daily from Sept. 1928 to Jan. 1929, since then with copper sulfate (until May 16). Tissue taken May 23, 1929.

Case 2. A. L., female, about 7 years old. Has been in the school 2 years. Had advanced trachoma when she came. Scar tissue present. Treatment as in Case 1. Tissue taken May 23, 1929.

Case 3. N. T. Y., male, about 8 years old. Has not been in the school and has had no treatment. Mostly scar tissue; few follicles along edge of tarsus. Portion of tissue showing follicles taken.

Case 4. S., male, 7 years old. Conjunctivae covered with follicles, not much scar tissue. Has not been in the school and has had no treatment. Duration of disease unknown.

Case 5. A. L., female, 12 years old. From St. Michael's school. Disease probably of 3 to 4 years duration. Upper lids show mostly scar tissue, but lower lids show follicles; tissue taken from lower lids. Treatment irregular.

Case 6. K. A., female, 10 years old. Trachoma of 5 years duration. Mostly scar tissue; few follicles. Treatment as in Case 1.

Case 7. L. W., female, 9 years old. Mostly scar tissue, few small follicles. Duration of disease unknown. Treatment as in Case 1.

The eyes were washed with sterile saline, and the follicles were removed by Dr. Richards after novocain anesthesia (2 per cent). The medium used was the same as that employed for cultivation from the monkey conjunctiva, except that some plates were made with human blood. The plates, which had been brought from New York in a humidor and protected to some extent from drying by being strapped with adhesive tape, were again closed with adhesive after inoculation. They were transported to New York in the humidor and were 9 days old when examined.

Cultures of *Bacterium granulosis* were obtained from Cases 3 and 4, *i.e.*, from the two cases which had received no treatment. The human blood medium appeared to be as satisfactory as that made with horse blood. The cultures were typical of *Bacterium granulosis* morphologically and culturally and induced the characteristic conjunctival lesions in monkeys (Table 6). Subsequently their action on carbohydrates was determined and found to conform with that of the Albuquerque Strain 1 of Noguchi.

(b) *Santa Fé and Albuquerque Cases*.—A second expedition to the Indian Schools was made in September, 1929. We are indebted to the Indian Service for providing facilities for the preparation of culture medium at the U. S. Indian Hospital in Santa Fé. No advanced untreated cases of trachoma were available for cultural study either at Santa Fé or at Albuquerque, and experiments with treated cases

TABLE 5

Monkey No.	Date	Culture inoc.		Incubation period	Result		Remarks
		Age	Temp.		Left	Right	
	1929	days		days			
{ P-1	June 15	57	4°-6°C.	73	+++	+++	Lesions persisting (8 months)
{ P-2	"	"	"	"	+++	+++	
{ P-3	"	"	"	"	+++	+++	
{ P-4	Sept. 28	162	4°-6°C.		—	—	
{ P-5	"	"	"		—	—	
{ P-6	"	"	"		—	—	
	1930						
{ P-7	Feb. 10	297	4°-6°C.		—	—	
{ P-8	"	"	"	8	++	++++	
{ P-9	"	75	Room		—	—	
{ P-10	"	"	"	8	+	+	

proved negative. It is of interest in this connection that a recent report of the Indian Service (10) shows a marked decline in the incidence of trachoma among the New Mexico (Pueblo) Indians, owing apparently to the systematic treatment which is being carried out in the Pueblo villages.

(c) *Leupp Cases*.—Through the kindness of Doctors Proctor and Richards, in cooperation with Commissioner Rhoads, of the Indian

TABLE 6

Inoculation of Bacterium granulosis from Fort Defiance Cases

Monkey No.	Inoculated		Incubation period	Result		Remarks
	Date	Material		Left	Right	
	1929		days			
F. D. 1	June 10	Cultures I gen. 7 days semi-solid II gen. blood agar 4 days, Case 3	77	+++	++++	Lesions progressed for 3 months. Discarded 5 months
F. D. 2	"	"		-	-	Reinoculated with pooled strains Cases 3 and 4 after 5 months. ++ lesions in 35 days
F. D. 3	"	"	64	+++	++++	Began to recede after 2 months. Discarded 7 months after inoc.
F. D. 4	"	"		-	-	
Chimpanzee "May"	"	"		-	-	Reinoculated with pooled strains Cases 3 and 4 after 5 months. Negative
F. D. 5	"	Similar cultures. Case 4	132	+++	+++	Lesions progressed for 2 months. Discarded 1 month later
F. D. 6	"	"	64	+++	++++	Lesions progressed for 3 months
F. D. 7	"	"	117	+	+	Transient lesions
F. D. 8	"	"		-	-	
F. D. 9	July 27	Case 4 cultures III, IV, V gen.		-	-	Discarded after months
F. D. 10	"	7, 14, 19, 25 days semisolid med. and blood agar		-	-	"
F. D. 11	"	"		-	-	"
F. D. 12	Nov. 11	Pooled cultures. Cases 3 and 4 10 days semi-solid	35	++	+++	
F. D. 13	"	"	35	+	+	Died 78 days after inoc.

TABLE 6—*Concluded*

Monkey No.	Inoculated		Incubation period	Result		Remarks
	Date	Material		Left	Right	
	1929		days			
Passage from F. D. 3						
F. D. 14	Aug. 16	Conj. susp. No. F. D. 3		—	—	
F. D. 15	"	"		—	—	
F. D. 16	"	"		—	—	
F. D. 17	"	"		—	—	

Service, a search for untreated cases of trachoma was made in the Navajo territory in Arizona, where systematic treatment has been less successfully applied because of the nomadic habits of the Navajos. It was found that at the Indian School at Leupp the treatment had not been carried out for at least 6 months, no physician having been on duty regularly. Thirteen cases were selected by Dr. Richards for study. Of these, 6 were cases of granular conjunctivitis of recent onset in which the diagnosis was not altogether certain, but in which there had been no treatment whatever, 5 were unquestionably trachoma, and 2 showed evidence of the beginning scar tissue formation, indicating that they were in all probability trachoma. The clinical data are presented in Table 7.

The follicular material used for inoculation of the culture media was removed by Dr. Richards, the eyes being first washed with sterile saline and the lids anesthetized with 0.5 per cent novocain. The usual medium was employed, *i.e.*, blood agar plates containing horse or human blood and a mixture of carbohydrates. The latter ingredient was the mixture originally used by Noguchi (1). It had been sterilized fractionally for 10 minutes on 3 successive days at 103°C. in a pressure cooker. There was some sedimentation on cooling, and only the clear supernatant fluid was added to the medium. The plates, which were prepared in Santa Fé the day previous to their use in Leupp, were strapped with adhesive tape after inoculation and brought back to New York in a humidor. They were not opened until arrival in New York, 9 days after inoculation. The weather conditions on the day of inoculation were unfortunate, in that high winds prevailed, and much sand and dust blew in through the windows and skylight of the operating room, where the cultures were made, hence the plates were in most cases nearly

overgrown with molds by the 9th day. A few, however, were less seriously contaminated, and colonies of *Bacterium granulosis* were found to be present. The follicle suspensions were brought back to New York and fresh plates inoculated.

TABLE 7

Case No.	Patient	Age	Diagnosis	Remarks	Culture
1	E. L.	7	Trachoma. Beginning scar tissue	Fairly recent case. Treated last year	—
2	M. N.	9	Doubtful (<i>i.e.</i> , no scar tissue)	May be beginning trachoma. Treated last year	—
3	P. T.	7	"	May be beginning trachoma. Treated last year	—
4	F. C.	14	Unquestionably trachoma. Scar tissue present	Old case. No treatment since last May	+
5	J. D.	10	"	Old case. Treated last year	+
6	H. W.	6	Recent case; some evidence of beginning scar tissue formation	Treated last year	+
7	M. C.	9	Recent case; some evidence of beginning scar tissue. Follicles in left eye only	Treated last year	—
8	B. H.	9	Unquestionably trachoma. Scar tissue present	No treatment since last May	—
9	H.	6?	Doubtful (<i>i.e.</i> , no scar tissue)	No treatment	+
10	I. M.	6?	"	" "	—
11	K. W.	7	"	" "	—
12	M. (Keems Canyon)	9	Unquestionably trachoma. Scar tissue present	" "	—
13	E. M.	10-11	Doubtful (<i>i.e.</i> , no scar tissue). Right eye only affected	" "	—

From the 4 cases which were regarded as unquestionably trachoma, *Bacterium granulosis* was obtained in 3. The organism was also obtained from Case 6, which showed evidence of early scar tissue forma-

tion, and from Case 9, which showed no scar tissue. The results of inoculation of the pooled cultures from Cases 5, 6, and 9 are recorded in Table 8. The lesions in Monkey L-6 are shown in Fig. 1.

4. Cultural and Biological Characteristics of the Various Strains

(a) *Viability*.—*Bacterium granulosis* remains viable for long periods on the semisolid ("leptospira") medium, and cultures 6 months old, kept at room temperature, with no other protection than the

TABLE 8
Inoculation of Bacterium granulosis from Leupp Cases

Mon- key No.	Inoculated		Incuba- tion period	Result		Remarks
	Date	Material		Left	Right	
	1929		days			
L-1	Nov. 22	Pooled cultures Cases 5, 6, and 9, I-II gen. semisolid med., blood agar plus carbohy- drates, and hor- mone agar plus carbohydrates 48- 72 hrs. old		—	—	
L-2	"	"	13	++	++++	++++ at 74 days
L-3	"	"	13	++	++	++ " " "
L-4	"	"		—	—	Died 13 days after inoc.
L-5	"	"	13	++++	++++	++++ at 74 days
L-6	"	"	13	++++	++++	++++ " " "

cotton plug, are readily transplantable. Even on a plain agar slant, on which the organism grows less readily, it has been found to remain transplantable for at least 49 days. It is probable that the bacterium lives as long as the medium contains a certain amount of moisture. Cultures kept in sealed ampoules for periods of 6 weeks to 10 months at 4° to 6°C., and for 69 days at room temperature, have proven viable and infective (Table 5).

(b) *Cultural Characteristics*.—The cultural characteristics of the strains of *Bacterium granulosis* thus far isolated have been practically

constant. In Tables 9 and 10 are shown the results of fermentation tests of the original strain and of the six strains isolated by us from Indian trachoma, as well as seven strains isolated by Finnoñ and Thygeson (3) and one by Kendall (5), which these workers kindly sent us.

The tests recorded in Table 9 were made on Hiss serum water medium containing 1 per cent of the respective carbohydrates and litmus (about 0.01 per cent of a 25 per cent solution) as indicator, to which were added 10 per cent horse serum and 0.2 per cent horse hemoglobin; those shown in Table 10 were made on Dunham's peptone water containing 1 per cent carbohydrate and 1 per cent Andrade indicator (0.5 per cent acid fuchsin to which normal sodium hydroxide is added until the dye is decolorized). With the latter media a definite acid reaction is evident in 3 to 5 days, the lactose being the only one of the carbohydrates affected which shows no reaction until after this period. The use of Durham tubes shows that no gas is formed in any of the carbohydrate media.

Bacterium granulosis reduces nitrates to nitrites but forms no indole. It does not liquefy gelatin.

It has been found that after a few subcultures, all the strains of *Bacterium granulosis* grow well on nutrient agar (veal infusion agar containing 1 per cent of Witte's peptone and 0.5 per cent sodium chloride). The medium must be freshly prepared. Growth is more abundant if the agar is enriched by the addition of a carbohydrate mixture and horse blood or rabbit hemoglobin. A number of carbohydrates has been added individually to the horse blood agar medium in a concentration of 2 per cent; of these, media containing dextrose gave the best growth.

(c) *Reaction to Gram's Stain*.—Since *Bacterium granulosis* failed to retain an appreciable amount of the Gram stain (Sterling's gentian violet) when decolorized for 2 minutes and counterstained with dilute fuchsin, Noguchi regarded it as Gram-negative. It may be noted that Morax (11) regards it as Gram-positive, and since he apparently does not make use of a contrast stain in his technique, his point of view is comprehensible. However, after a contrast stain, even one as weak as Bismarck brown, no Gram stain can be detected in young, actively growing cultures. In old cultures, or cultures grown under unfavorable conditions (e.g., in dextrose broth), numerous bizarre forms are seen which may retain the Gram stain. A form with an equatorial

swelling, suggesting a central spore, is not uncommon in such cultures and usually stains Gram-positive, especially in the central portion.

(d) *Effect of Heat.*—*Bacterium granulosis* is rather sensitive to heating, being killed by exposure for 10 minutes to a temperature of 57°C. The thermal death point was tested by submerging sealed ampoules

TABLE 9
Fermentation Tests. Hiss Serum Water Medium

	F. D. 3	F. D. 4	L. 5	L. 6	L. 9	Kendall
Dextrose.....	+	+	+	+	+	+
Levulose.....	+	+	+	+	+	+
Mannose.....	+	+	+	+	+	+
Saccharose.....	+	+	+	+	+	+
Raffinose.....	+	+	—	+	+	—
Inulin.....	+	+	—	—	—	+
Galactose.....	+	+	+	+	+	+
Maltose.....	+	+	+	+	+	+
Salicin.....	+	+	+	+	+	+
Xylose.....	+	+	+	+	+	+
Mannitol.....	+	+	+	+	+	+
Dextrin.....	+	+	+	+	+	+
Arabinose.....	+	+	+	+	+	+
Amygdalin.....	+	+	+	+	+	+
Lactose.....	+	+	+	+	+	±
Dulcitol.....	—	—	—	—	—	—
Rhamnose.....	—	—	+	+	+	+
Trehalose.....	—	—	+	—	—	+
Sorbitol.....	—	—	—	—	—	—
Inositol.....	—	—	—	—	—	—
None.....	—	—	—	—	—	—

+, color of indicator changed to red. No coagulation.

—, no change in color of indicator.

Readings recorded were made at 9 days. No change occurred after this time, and the tubes were discarded at 14 days.

F. D. = Fort Defiance. L. = Leupp.

containing 0.5 cc. of a rich culture (5 days old on the semisolid medium) in water at temperatures varying from 52°C. to 65°C., and 5 and 10 minute exposures were made at each temperature. The whole amount of the heated culture was transferred, immediately after the heating, to fresh semisolid medium.

TABLE 10
Fermentation Tests, *Dunham's Peptone Water Medium*

	Aq. 1	D. 1	ID. 2	D. 4A	D. 5	D. 6	D. 7	F.D. 4	L. 4
Dextrose.....	+	+	+	+	+	+	+	+	+
Levulose.....	+	+	+	+	+	+	+	+	+
Mannose.....	+	+	+	+	+	+	+	+	+
Saccharose.....	+	+	+	+	+	+	+	+	+
Raffinose.....	-	-	-	-	-	-	-	-	-
Inulin.....	+	±	+	±	+	±	+	±	+
Galactose.....	+	+	+	+	+	+	+	+	+
Maltose.....	+	+	+	+	+	+	+	+	+
Salicin.....	+	+	+	+	+	+	+	+	+
Xylose.....	+	+	+	+	+	+	+	+	+
Mannitol.....	+	+	+	+	+	+	+	+	+
Dextrin.....	+	+	+	+	+	+	+	+	+
Arabinose.....	+	+	+	+	+	+	+	+	+
Amygdalin.....	+	+	+	+	+	+	+	+	+
Lactose.....	+	+	+	+	+	+	+	+	+
Dulcitol.....	-	-	-	-	-	-	-	-	-
Rhamnose.....	+	+	+	+	+	+	+	+	+
Trehalose.....	+	+	+	+	+	+	+	+	+
Sorbitol.....	-	-	-	-	-	-	-	-	-
Inositol.....	-	-	-	-	-	-	-	-	-

Aq. = Albuquerque.

D. = Strains isolated by Thygeson in Denver.

F. D. = Fort Defiance.

L. = Leupp.

(e) *Effect of Cocaine*.—The organism is also sensitive to the action of cocaine hydrochloride, being killed in 5 minutes by 4 and 5 per cent solutions, and in 15 minutes by weaker solutions (2 and 3 per cent). Similar concentrations of procaine hydrochloride (novocain) have no inhibitory effect on its growth. In obtaining material for cultivation, therefore, it is desirable to avoid long exposure to high concentrations of cocaine.

SUMMARY AND CONCLUSIONS

One of the strains of *Bacterium granulosis* isolated by Noguchi in 1926 has been maintained in culture and in monkeys and continues to be capable, after 3 years, of inducing a chronic granular conjunctivitis in monkeys. Cultures of this strain have been recovered from the monkey lesions as late as 3 years after inoculation and have been shown to reproduce the granular disease in monkeys.

Six additional strains of *Bacterium granulosis* have been isolated from cases of trachoma occurring in the Indian schools of Arizona. The cultures thus obtained are identical morphologically and culturally with those isolated by Noguchi and have induced the same chronic granular conjunctivitis in monkeys. Advanced untreated cases are more favorable for cultural study than treated cases.

Cultures of *Bacterium granulosis* kept on semisolid medium containing 10 per cent rabbit serum ("leptospira medium") remain viable for many months at room temperature, and sealed ampoules of such cultures have been found to retain their pathogenicity for the monkey conjunctiva for at least 69 days at room temperature and at least 284 days at 4° to 6°C.

Several additional cultural and biological characteristics of *Bacterium granulosis* have been described. Of outstanding importance is the fact that cocaine, in contradistinction to novocain, has a bactericidal effect on the organism. This fact, in view of the common use of cocaine for anesthesia, may explain the negative results of cultivation experiments reported by some workers.

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EXPLANATION OF PLATE 26

FIG. 1. *M. rhesus* L-6, 19 days after inoculation with pooled cultures from Leupp cases.

FIG. 2. *M. rhesus* 80, 2½ years after inoculation (1), showing presence of scar tissue.

FIG. 3. *M. rhesus* 5, 1½ years after inoculation, when the lid showed renewed activity of the lesions, and the left cornea became diffusely cloudy.

FIG. 4. *M. rhesus* 61, 57 days after inoculation with routine subcultures of Albuquerque Strain 1.

FIG. 5. *M. rhesus* 51, 65 days after inoculation with cultures recovered from *M. rhesus* 5.

Right

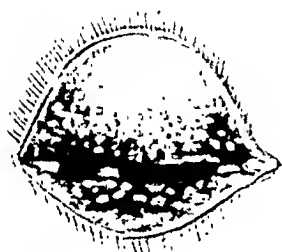
Left



1



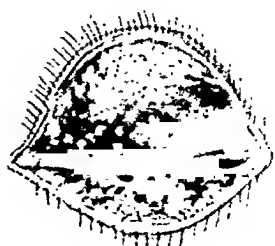
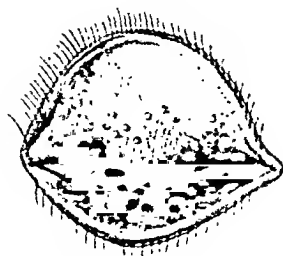
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3



4



5



THE TRANSMISSION OF PERIODIC OPHTHALMIA OF HORSES BY MEANS OF A FILTERABLE AGENT

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PLATES 27 TO 31

(Received for publication, June 2, 1930)

In a previous communication (1) one of us together with Burky reported the results of a bacteriological study of periodic ophthalmia in horses. Both aerobic and anaerobic cultures of eyes freshly enucleated from horses with both active and quiescent periodic ophthalmia, were uniformly negative. The disease, however, has been shown by others to be definitely transmissible by inoculation into a normal eye of an exudate from an eye that was the seat of active disease. The purpose of this paper is to report the pathology of the disease as observed in the epidemic which we have investigated and to publish observations indicating that in this particular epidemic the disease was caused by a filterable agent.

In the fall of 1926 there occurred a sudden outbreak of periodic ophthalmia among thorough-bred horses on a farm located in high-rolling country in northern Maryland. The clinical appearance of the disease in this epidemic was identical with that observed elsewhere, and did not differ from that seen in sporadic cases which have come to our attention. The disease is essentially a recurrent serous uveitis. The onset is characterized by lachrymation, oedema of the periorbital fold, slight haziness of the cornea, the occurrence of a gelatinous, mucopurulent exudate in the anterior chamber, and the usual signs of iritis with synechiae and exudations into the vitreous. Exudates in the retina occurred, but were difficult to study on account of the disease of the anterior portions of the uvea.

The initial attack usually lasts from 4 to 10 days and then subsides, resulting at first in little permanent damage. The period of remission

is irregular, varying usually from 3 to 6 weeks, after which a fresh attack occurs. The attacks recur one after the other with increasing or decreasing severity. In a few horses the eyes recover from the attacks and show only small vitreous opacities, occasional posterior synechiae and varying amounts of retinal atrophy. In a few of the horses in which such recovery occurred there was almost complete retinal atrophy with resultant blindness, while in others the only evidence of the disease was limited to several vitreous opacities. The commonest outcome, however, is for the attacks to occur with increasing severity, with the formation of complete posterior annular synechiae, secondary glaucoma, cataract and ultimately moderate phthisis bulbi.

Pathology

The pathology of the disease has been described by several authors, and most recently by Heinrich Jakob (2). These descriptions, however, do not correspond exactly with the picture we have constantly observed in the horses studied.

The characteristic lesion we have observed appears to be a focal mononuclear infiltration of the uveal tract, the pigment epithelium, the retina and the optic nerve.

This focal infiltration spreads, cyclitic membranes may form, and scarring with complete detachment of the retina may take place. The mononuclear infiltration occurs throughout the optic nerve and in focal points throughout the retina. In advanced, severe cases a heavy cyclitic membrane covers the ciliary process. The cyclitic membrane is densely infiltrated with small round cells. Follicular accumulations of round cells, which superficially resemble lymph follicles, are found throughout the stroma of the uveal tract and in the cyclitic membrane. The essential features of the pathological picture are illustrated in the micro-photographs.

Figs. 1, 2, 3, 6, 7, 10, 11 show, under different magnifications, typical lesions from a number of different horses whose vision was destroyed by the natural disease.

One of the most notable features in the pathological picture is the formation of follicular collections of round cells, which superficially resemble lymph-follicles. These may occur in the stroma of the iris, or outside the normal tissues of the eye, lying in the inflammatory cyclitic membrane. Fig. 12 shows the typical appearance of these follicular collections of cells. While these collections superficially resemble lymph-follicles, they appear, on closer inspection, to differ from them. They have no germinal centers; capillaries are present, and both large and small mononuclear

cells are found, together with plasma and epithelioid cells. Further, we could find no sinuses, such as are found in true lymph-follicles. Stained with the Foot stain, the reticulum characteristic of true lymph-follicles could not be demonstrated.

Whether the follicular collections of cells are pathognomonic of this disease cannot be said as yet. They have been constantly observed in all the horses with periodic ophthalmia that we have studied, and we have not found them in the eyes of horses with uveitis caused by bacteria. Further study of uveitis in horses produced by other agents will be necessary to determine whether or not the collections of cells are to be considered as a specific feature of periodic ophthalmia.

EXPERIMENTAL

Our first studies on the etiology of this disease were concerned with an attempt to cultivate a bacterium from the diseased eyes. All aerobic and anaerobic cultures from eyes showing the disease in both active and quiescent form were entirely negative. We were forced therefore to disagree with Rosenow's (3) conclusion that a Gram negative flavobacterium is the etiological agent of the disease. After numerous attempts to demonstrate other possible causes such as spirochetes, plasmodia, and tuberculin hypersensitivity, none of which were successful, we considered the possibility of a filterable agent as the specific etiologic factor.

A horse in the acute stage of the disease was sacrificed and the eyes removed. One eye was saved for histological study, and the other, affected with the disease in the acute stage, was opened under aseptic precautions, the aqueous and vitreous humors as well as the retina and uveal tract removed and ground up in a sterile mortar with sterile sand and several cubic centimeters of physiological salt solution. The supernatant fluid was then passed through a Berkefeld N filter. This filtrate was tested for sterility by aerobic and anaerobic cultures on rabbit blood agar, glucose beef infusion broth and cooked meat medium under vaseline seal. Under butyn anaesthesia 0.5 cc. of this filtrate was then injected into the vitreous humor of a normal horse. Within 24 hours the horse developed the clinical picture typical of periodic ophthalmia, with oedema of the periorbital fold, lachrymation, photophobia, ciliary congestion, exudation in the anterior chamber, vitreous opacities and the evidence of an acute iritis. The attack lasted about 10 days, then subsided and recurred several times.

This experiment indicated that it was possible to recover from the eyes of horses with periodic ophthalmia a filter-passing agent which

was capable of producing the clinical picture of the disease in a normal horse, but gave no indication as to whether or not the agent was a living virus. In order to throw light on this point it was decided to attempt to transmit the disease to other species with the hope of carrying it through several transfer-generations of animals, because of the expense entailed in the use of horses. Rabbits were selected for this purpose, and a second horse with periodic ophthalmia in the acute stage was sacrificed and the eyes enucleated. One eye was saved for histological study and a filtrate was prepared from the second eye in the manner described above. Inasmuch as the same technique for preparing and testing the filtrate for sterility was used throughout all the transmission experiments we shall, for the sake of brevity, refer to these procedures as the "usual technique." The clear filtrate, proven sterile by culture, was injected directly into the vitreous of each eye of a series of six rabbits. All injections were made while the rabbits were under the influence of an anaesthetic.

There quickly developed in these rabbits a clinical picture which, while not similar in every respect to that observed in horses, was nevertheless quite constant.

There was no visible involvement of the anterior uvea, but within 24 hours there developed an acute retinitis with fluffy exudates throughout the retina. In some of the animals the lesions tended to recur after a remission of 2 to 3 weeks. The histology of these lesions is described below. Control inoculations into normal rabbits' eyes with filtrate of emulsions from normal horses' eyes failed to produce such lesions, and aerobic and anaerobic cultures of the diseased eyes were sterile.

These facts warranted the surmise that the disease had been transmitted successfully to rabbits by the intra-vitreous injection of a filtrate from the eyes of a horse with periodic ophthalmia.

Acting on this assumption, an attempt was next made to carry the disease from the rabbit back to the horse.

A normal horse (No. 2) was inoculated directly into the vitreous with 1 cc. of a filtrate prepared according to the usual technique from one diseased eye of each of three of the experimentally infected rabbits. Within 24 hours after inoculation the horse showed the typical picture of periodic ophthalmia observed in horses with the natural disease. The acute symptoms persisted for 10 days and then subsided. 3 weeks later the disease recurred and there was subsequently a second recurrence.

This experiment suggested that the filterable agent was capable of propagation in the rabbit's eye, but as the transmission had been made through a single transfer-generation of rabbits from the naturally diseased horse to the experimentally infected eye of the horse, it was necessary to elaborate the original experiment in order to establish this theory.

An attempt was made therefore to transmit the disease from horses to rabbits and to carry it through several transfer-generations of rabbits in succession before making the crucial experiment of transmitting it to the horse.

Accordingly another horse with periodic ophthalmia was sacrificed and a filtrate prepared with the usual technique from the acutely diseased eye. This filtrate, proven sterile by aerobic and anaerobic cultures, was injected into the vitreous of each eye of a series of four rabbits. All four rabbits developed a retinitis similar to that observed in the preceding experiments. After an interval of 2 weeks, two of these rabbits were sacrificed, the eyes removed, cultured by both aerobic and anaerobic methods, and a filtrate prepared from them. A second series of four rabbits was inoculated by intravitreal injection with this filtrate. The remaining two rabbits were kept for further observation and for histological study.

In this manner the disease was carried through six successive transfer-generations of rabbits with the constant occurrence of the typical retinitis. Aerobic and anaerobic cultures were made of all eyes removed for preparation of filtrates. These cultures were uniformly negative. Occasionally a rabbit was encountered which showed no lesions after inoculation. Such rabbits were not used for transfer. These rabbits which appeared refractory to inoculation were the exception, for the occurrence of the retinitis was remarkably constant throughout.

A filtrate from the eyes of three rabbits of the sixth transfer-generation was then inoculated into the vitreous of the left eye of each of two normal horses (Nos. 3 and 4). Both of these horses developed a clinical picture similar to that observed in the natural disease.

The initial symptoms were oedema of the periorbital fold, lachrymation, photophobia, ciliary congestion and the occurrence of a gelatinous, mucopurulent exudate in the anterior chamber. The signs of inflammation were noted 24 hours after inoculation, and they reached their height within 48 hours. The initial attack lasted from 1 to 2 weeks and then diminished, leaving only slight evidence

of inflammation. In each horse the disease recurred after periods of from 2 to 4 weeks. One horse (No. 3) was apparently very susceptible. The injected eye showed an unusually severe reaction. The attacks recurred with increasing severity, and the final picture was similar to that observed in cases of severe natural disease—complete posterior synechiae, cataractous lens, and moderate phthisis. The other horse (No. 4) had four attacks, each less severe than the preceding one. The attacks finally ceased spontaneously, and the picture was then similar to that observed in the eyes of horses in which the natural disease had healed normally, as the only evidence of the disease was some clouding of the vitreous and a few posterior synechiae. None of the experimental horses had any symptoms of involvement of the control or uninjected eye.

Controls

The following control experiments were performed.

1. A filtrate was prepared in the usual manner from the eye of a normal horse and injected into the vitreous of each eye of four rabbits. The eyes of these animals did not exhibit the retinitis observed in the eyes of rabbits injected with filtrates from the eyes of horses with periodic ophthalmia. Histological study of these eyes showed no pathological lesions.

2. The eye of a normal horse was injected with 2 cc. of sterile filtrate obtained from normal rabbits' eyes. This horse showed a moderate reaction following the injection which lasted about 48 hours. Within 4 days the eye appeared entirely normal, and remained so for a period of 3 months observation. The horse was then sacrificed. Histological examination of the inoculated eye showed no pathological lesions resembling, even remotely, those of periodic ophthalmia.

3. In order to exclude a possibility that the experimental lesions were due to traumatism alone, salt solution was injected into the vitreous of the eyes of normal rabbits and of a normal horse. Clinical and histological examination of these eyes revealed no pathological lesions.

Pathology in Experimental Rabbits

The pathological lesions found in the experimental rabbits were less extensive than those found in the horses. They consisted chiefly in focal areas of acute retinal inflammation with disorganization of the normal retinal picture by round cell infiltration, which was fundamentally the same as the pathological lesion found in horses. No follic-

ular collections of cells were observed. These lesions were seen both in the acute stage and the stage of repair. Figs. 8 and 9 show such lesions. Occasionally lesions in the choroid were found, although as a rule the uveal tract was remarkably free from abnormalities.

The optic nerve was also involved in a few rabbits showing mononuclear infiltration of the stroma and of the tissues over the surface of the nerve. Fig. 13 illustrates such lesions.

One rabbit showed a definite infiltration in the sheath of the optic nerve, such as is found in meningitis, the cells near the nervehead being chiefly mononuclear in type, while polynuclear cells were found posteriorly in the sheath of the nerve (Fig. 4).

We have as yet made no intra-cerebral injections of this filterable agent, and the brains of the experimental rabbits and horses have not yet been studied, so that we have no information as to the condition of the central nervous system.

Pathology of Disease in the Inoculated Horse

The lesions in the disease produced experimentally were uniformly of the same character as those found in the natural disease.

Figs. 5 and 14 to 17 illustrate the various pathological lesions found in Horse 3 in which the attack recurred with increasing severity until the disease reached the end stage: mononuclear infiltration of the iris with collections of round cells (Fig. 14); acute lesions of the choroid and retina with proliferative changes, and disorganization and infiltration of the retina by mononuclear cells (Fig. 15); follicular-like collections, chiefly of mononuclear cells, lying both in the stroma of the anterior uvea and in the cyclitic membrane (Figs. 16 and 17) and optic neuritis with mononuclear infiltration of the nerve stroma (Fig. 5).

The eyes of the other two horses (Nos. 2 and 4) in which the clinical picture of the disease was produced experimentally, finally healed, and at the time the horses were sacrificed the eyes showed but little clinical abnormality. Pathological study of the eyes of these horses, as might be expected, did not show the pronounced changes found in the eyes which were the seat of advanced disease.

One horse (No. 2) showed several focal areas of round cell infiltration throughout the stroma of the anterior uvea, while the other horse (No. 4) showed some diffuse cellular infiltration around the pectinate ligament and the root of the ciliary processes. We have, unfortunately, no histological preparations of eyes from horses that have recovered from the natural disease, but clinical examination of such eyes gives no reason to suppose that more extensive pathological lesions could be found than these illustrations show. The lesions found in these two

horses illustrated in Figs. 18 and 19, were what might be expected in horses in which the disease had healed after several acute attacks.

COMMENT

These experiments were designed to solve the problem of whether periodic ophthalmia in horses can be successfully transmitted by means of a filter-passing agent. We believe it has been. The reasons upon which we base our belief are three-fold. First, the experimental disease is indistinguishable clinically from the disease as it is observed in nature, not only in the actual appearance of the lesions but in the relapsing course which they pursue. Secondly, the pathological lesions in the experimental disease and in the natural disease are identical. Thirdly, we could find no evidence that bacteria produced these lesions. Aerobic and anaerobic cultures made from eyes in the acute stage of the disease gave uniformly negative results, and sections of such eyes, stained by special methods did not show either bacteria or spirochetes. Furthermore, the infiltration with mononuclear cells, and the almost complete absence of polymorphonuclear cells in the exudates would be unusual in a disease caused by bacteria such as are usually found in pyogenic inflammations. We believe therefore these experiments to indicate that this epidemic of periodic ophthalmia in horses is caused by a filterable agent, and that the disease can be transmitted to other animals by the intra-vitreous inoculation of this agent. The successful transmission of the disease to the horse after successive transfers through six series of rabbits indicates that the agent is capable of propagation in this species and belongs to the group of filterable viruses. In the main this agent appears to attack the optic nerve, retina, pigment epithelium, and the uveal tract. Whether the involvement of the uveal tract is an extension of the inflammation from the retina and pigment epithelium of the ciliary processes cannot be stated. In rabbits the disease produced by this filterable agent is limited almost entirely to the nervous structures of the eye, while in horses the onset and involvement of the anterior uvea are so sudden and abrupt that it is impossible to tell where the initial lesion starts.

It is also by no means certain that the action of this filterable agent is limited solely to the eyes. Two horses and a number of rabbits

were autopsied and sections of the viscera studied histologically, but no lesions were found. The brains and spinal cords, however, were not examined in any of the animals. Sections of the optic nerves, both of the horses and rabbits, suggest the possibility that disease of the central nervous system may also occur. None of the horses under observation died from natural causes, but there was a low mortality among the experimental rabbits. It has been noted that horses affected with the disease appear irritable and tend to throw their heads up unduly. The rabbits were observed at regular intervals, but we did not see in them any symptoms of central nervous system involvement. We can therefore hazard no opinion as to this possibility, other than that the sections of the eyes suggest that there may have been such involvement. This is a point for future investigation.

We have not been able to demonstrate inclusion bodies. In a number of sections we have found granules in cells which suggested inclusion bodies, but in no instance could these be definitely identified as such. An explanation of our failure to find such bodies may be that they are found chiefly in the early stages of diseases caused by filterable agents; our sections of eyes with the natural disease were all obtained from blind horses in which the disease had reached the end stage. Likewise, our experimental horses were all in the late or healed stages of the disease at the time of autopsy, for in the transmission experiments it was necessary to observe the horses for a long period of time in order to establish the clinical picture of recurrent attacks.

Not all horses appear uniformly susceptible to the disease. The morbidity rate in a herd is relatively low. There was a marked variation in the severity of the clinical picture both in the natural disease and in that experimentally induced. While the eyes of a few horses affected with the natural disease eventually healed with little or no final damage to the eyes, the disease usually progressed until there was more or less complete scarring of the eyes with concomitant loss of vision. The comparative mildness of the experimental disease in a few instances may possibly be attributable to a partial natural immunity. The low morbidity of the disease in a herd is in harmony with this conception. Moreover, not all rabbits appear to be susceptible to the experimental disease. A few were encountered which showed no reaction to the intra-vitreous injection of the filterable agent.

We have no knowledge of the mode of infection or the natural method of transmission of the disease. Intravenous inoculation of filtrates into four rabbits gave negative results. We cannot state whether or not this filterable agent is similar to any of the other filterable viruses so far studied, or if it constitutes a specific entity in itself. We have as yet made no studies on the possible protective or therapeutic properties of convalescent serum. These are all points for further investigation.

CONCLUSIONS

A filterable agent has been obtained from the humors and tissues of the eyes of horses suffering from active periodic ophthalmia. The intra-vitreous injection of this filtrate produced in normal horses the same clinical and pathological picture observed in the natural disease. This filtrate injected into rabbits produced a different clinical picture, but the essential pathological lesions closely resembled those found in horses. After passage of the filterable agent through six generations of rabbits, it again produced the clinical and pathological picture of the natural disease when injected into the eyes of normal horses.

It appears, in this epidemic at least, that this filterable agent was the specific etiological factor of the periodic ophthalmia.

In conclusion we desire to express our thanks to Major G. L. Stryker for the gift of horses with the disease, and for his cooperation and assistance throughout the course of this investigation, without which it could not have been conducted. We are indebted to Dr. F. B. Kindell and Dr. S. S. Blackman for autopsy examination of horses, to Dr. Jonas Friedenwald and Dr. F. H. Verhoeff for their valuable assistance in the examination of the histological specimens of the eyes, and to Dr. T. B. Turner for aid in the manipulations incident to transfer.

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EXPLANATION OF PLATES

PLATE 27

- FIG. 1. (Natural disease.) Mononuclear infiltration of stroma of iris and follicular accumulation of round cells (high power)
FIG. 2. (Natural disease.) Monocellular infiltration over ciliary processes.
FIG. 3. (Natural disease.) Optic neuritis.
FIG. 4. (Experimental disease in rabbit.) Mononuclear infiltration in sheath of optic nerve.
FIG. 5. (Experimental disease in Horse 3.) Optic neuritis with mononuclear infiltration of nerve stroma.

PLATE 28

- FIG. 6. (Natural disease.) Focal lesion in retina; mononuclear infiltration.
FIG. 7. (Natural disease.) Disorganization and atrophy of retina in advanced disease.
FIG. 8. (Experimental disease in rabbit.) Acute lesion of retina.
FIG. 9. (Experimental disease in rabbit.) Healing lesion of retina with fibrosis.

PLATE 29

- FIG. 10. (Natural disease.) Acute lesion over pigment epithelium and follicular accumulation of mononuclear cells.
FIG. 11. (Natural disease.) Mononuclear infiltration of choroid.
FIG. 12. (Natural disease.) Follicular accumulation of mononuclear cells (low power).
FIG. 13. (Experimental disease in rabbit.) Optic neuritis.

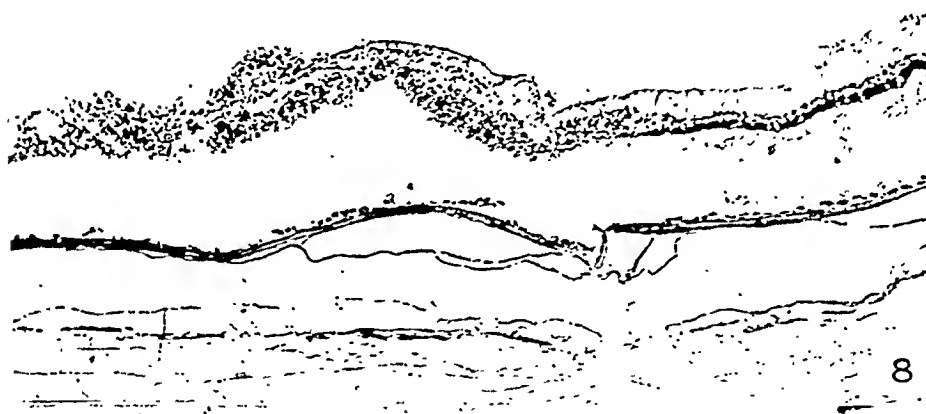
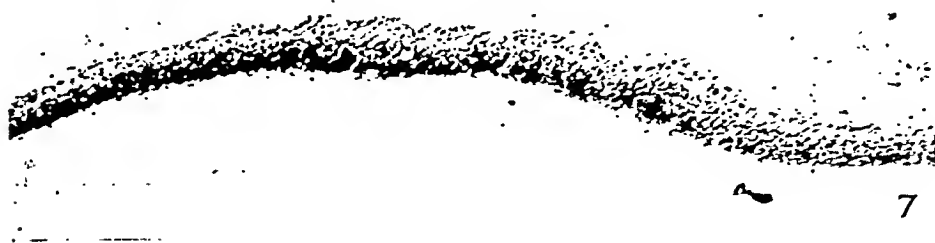
PLATE 30

- FIG. 14. (Experimental disease in Horse 3.) Mononuclear infiltration of iris and follicular collection of cells.
FIG. 15. (Experimental disease in Horse 3.) Acute lesion in choroid with dense mononuclear infiltration.
FIG. 16. (Experimental disease in Horse 3.) Infiltration and follicular accumulation of mononuclear cells in anterior uvea (low power).

PLATE 31

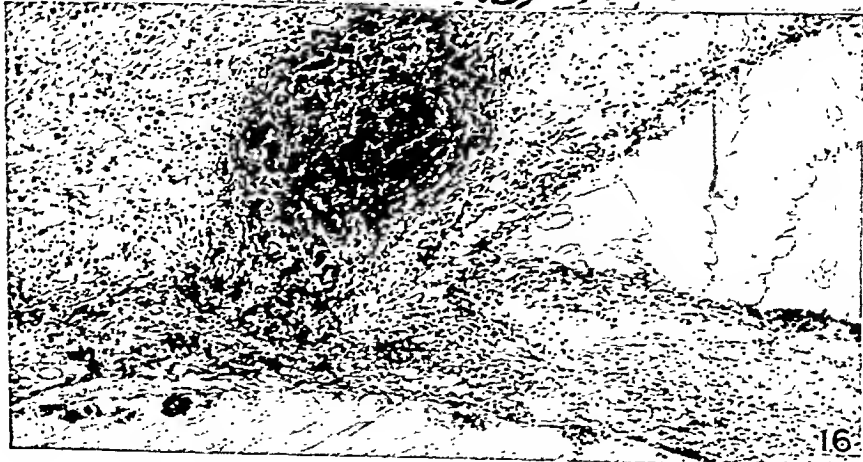
- FIG. 17. (Experimental disease in Horse 3.) Follicular-like collection of mononuclear cells lying in the inflammatory cyclitic membrane (low power).
FIG. 18. (Experimental disease in Horse 2.) Focal area of mononuclear infiltration in anterior uvea (high power).
FIG. 19. (Experimental disease in Horse 4.) Diffuse mononuclear infiltration about pectinate ligament.

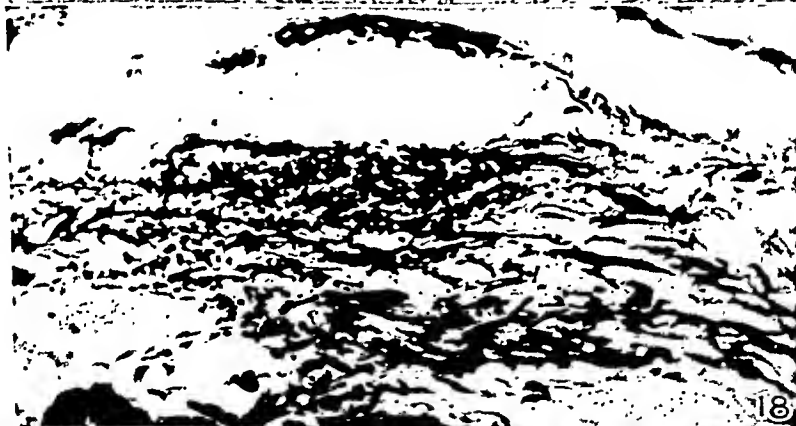
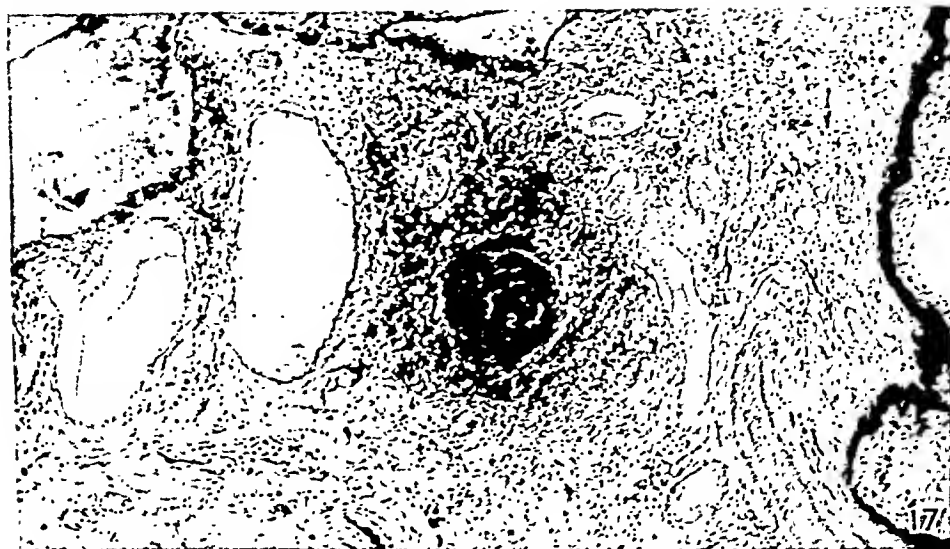












STUDIES ON TYPHUS FEVER

II. STUDIES ON THE ETIOLOGY OF MEXICAN TYPHUS FEVER

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PLATES 32 TO 34

(Received for publication, June 26, 1930)

In experimental typhus fever of guinea pigs the differences between the European strain and the Mexican strain are sufficiently definite to permit differentiation. The chief points which make such recognition possible were emphasized by Mooser (1) and have been confirmed in every particular by ourselves. They consist especially in the regularity with which testicular swelling occurs, after intraperitoneal inoculation, in the Mexican variety, the constant presence of the *Rickettsia*-like bodies described by Mooser in the tunica and the relatively earlier temperature rise which, in the Mexican virus animals, may begin on the fourth, fifth or sixth day, whereas in the European type it is usually deferred until the ninth day or later. In the European type, also, the temperature is apt to reach a higher point and, as Mooser correctly points out, the nodular lesions in the brain described by Otto and Dietrich (2), Spielmeier (3), Wolbach (4) and others are more frequent. It should be noted, however, that if the Mexican virus is subcutaneously injected instead of intraperitoneally, scrotal swelling with *Rickettsiae* does not appear, the temperature is apt to begin later and the characteristic brain lesions are present. Subcutaneously inoculated Mexican virus, then, produces a disease closely simulating the European variety. Moreover, even after intraperitoneal inoculation, certain strains of the Mexican disease (like the "J" strain described by one of us in a previous communication) will occasionally produce disease without tunica lesions and with temperature curves and other features closely analogous to the European type of disease.

Common to the two diseases is demonstrable louse transmission (5), cross immunization and, under proper conditions, the brain lesion. In the European disease, also, we have been able to confirm Pinkerton's (6) observation that *Rickettsia* bodies similar to those described by Mooser can be found if the examination is made at the proper time. Failure in finding them, we believe, has been due largely to the fact that search has been deferred until the temperature rise has begun, a period at which the organisms have disappeared from the tunica. Why it is so much more difficult to find *Rickettsiae* in the tunicas of the European variety, we have no idea. Summarizing all these facts, however, it seems reasonable to assume that we are dealing with two closely related varieties of a single disease group into which, also, Rocky Mountain spotted fever may be placed, Mexican typhus fever occupying a position, as far as guinea pig inoculation is concerned, midway between the European typhus and Rocky Mountain spotted fever.

This of course does not justify one in applying all facts discovered about Mexican typhus fever to the European variety, but it does render it extremely likely that there should be etiological analogy and that immunological observations made on one disease can probably be applied by proper experimental methods to the other.

The work recorded in the present paper has been done almost entirely with the Mexican variety and deals primarily with the etiological problem. The causative significance of *Rickettsiae* has been advocated by many observers in the past, has been rendered likely by studies with lice and has been considerably strengthened by the discovery of these organisms in the tunica of Mexican typhus animals by Mooser. It is further supported by the experimental louse transmissions recently reported by this writer with Dummer (5) in which lice were infected by feeding on monkeys inoculated with Mexican typhus and developed organisms indistinguishable from those seen in the guinea pig. Although the methylene blue-saffranin method has shown that these bodies stain as easily as do bacteria, there is no doubt of their identity with the Giemsa-staining *Rickettsiae*, because of morphological similarities, intracellular position and resistance to cultivation. It may well be that cultivation may be accomplished in the future, and if so, the *Rickettsiae* may be recognized as a specialized variety of true

bacteria which have been modified by normal habitat in insects. Until this is accomplished, however, the Mooser bodies must be regarded as true *Rickettsiae* and this class of organisms segregated for purposes of convenience.

In spite of the probability with which the etiological importance of these organisms has been indicated by many investigations of the past, the crucial etiological demonstration is still lacking and the literature is confused by suggestions of filterable virus, mutations of proteus organisms and the suspicion that possibly the *Rickettsiae* or Mooser bodies represent merely a concomitant infection carried along with the typhus virus. Objections to the acceptance of *Rickettsiae* etiology have been based largely upon the uniform failure of attempts to find the *Rickettsiae* in virulent blood, organs and brain lesions; and the usually small numbers of visible *Rickettsiae* in tunica lesions, together with their intracellular position, has made it impossible to carry out inoculation experiments with material that did not contain, in addition to *Rickettsiae*, blood plasma and cells. The results obtained in our own laboratory in the last few years, which we think have strengthened the probable etiological significance of the Mooser bodies, have consisted in negative filtrations, the relatively greater degree of virulence of material containing large numbers of these organisms, the encouraging results of active immunization with formalinized tunica and the typical infectiousness of lice injected by the Weigl method with spleen material in which no *Rickettsiae* could be found by direct examination. Also, one of us has recently shown that the tunica lesion could disappear for several inoculation generations only to come back from time to time in individual guinea pigs, in every case with the presence of *Rickettsiae*.

It is not impossible that the peculiar pathology of the disease in guinea pigs could be explained by the fact that, on intraperitoneal inoculation, the *Rickettsiae* accumulate in the tunica, perhaps because of favorable temperature conditions or nutritive and cellular selective advantages and, from there, penetrate the body generally, disappearing from the tunica as the disease becomes generalized.

The present paper is a record of experiments in which we have endeavored to increase the susceptibility of animals to Mexican typhus fever in order to investigate with greater accuracy the dis-

tribution of the *Rickettsiae* or Mooser bodies throughout the organs, and in order to obtain these microorganisms in sufficient numbers to enable us to carry out a crucial etiological experiment.

Our first attempts were based on the reasoning that the testis was of a lower temperature than the body as a whole. The supposition was that perhaps the organisms were carried through the peritoneum into the tunica, and there found lower temperature conditions. The experiments carried out on this plan consisted in the use of two methods to lower the temperature of guinea pigs—one by keeping them, after inoculation, in a cold room having an average temperature of 5° to 8°C.; the other in lowering the general temperature by feeding considerable amounts of methyl alcohol by mouth. The latter method was by far the more successful, actually bringing the temperature of guinea pigs down to 97° to 99° during a period of prolonged intoxication, and appearing to give us a much more plentiful *Rickettsiae* yield in the tunica. Neither of these methods, however, was adequate for our purposes.

While studying tunica lesions and exudates by the methylene blue-saffranin method, we frequently saw actual phagocytosis of *Rickettsiae* by polymorphonuclear neutrophils. A drawing of such a condition is shown in Fig. 1. This, we believe, accounts for the occasional slight infectiousness of washed leucocytes recorded in a previous paper (7). It also suggested to us that possibly the destruction of *Rickettsiae* in the body was actively carried on by the mobile phagocytes, and that an inhibition of phagocytosis might give us better results. In consequence, we turned to the use of benzol, as recommended by Weiskolten, Schwartz and Steensland (8).

We carried out experiments with benzol both in guinea pigs and in rats, injecting subcutaneously mixtures of equal volumes of benzol and olive oil and after several such treatments—or at the same time with these treatments—inoculating intraperitoneally with tunica material containing considerable numbers of the organisms.

The benzol injections have given us remarkable results in four or five animals up to the present time, with less successful experiments in a number of others. We have still a great deal to learn about the amounts of benzol that can safely be injected, the intervals of injection, and particularly the most advantageous time after adminis-

tration of the benzol at which the infectious material should be injected into the prepared animals. These points are being subjected to detailed study at the present time. Meanwhile, the successful animals have given us results which we believe to be of the greatest importance in connection with the etiology of typhus fever.

An example of one of the best of these benzol rat experiments is the following:

Experiment—Benzol Rat 1A.—This rat was subcutaneously injected on May 12 with 2 cc. of a mixture of equal parts of benzol and olive oil. 2 days later, on May 14, this animal received intraperitoneally an injection of tunica of Guinea pig 1, moderately rich in both intra- and extra-cellular *Rickettsiae*. On May 18, when the rat was to all appearances quite sick, it was chloroformed, carefully dissected and smears taken from the tunica, peritoneal exudate and peritoneal cells, spleen, liver, brain, bone marrow and blood. Examination of these smears showed enormous numbers of intra- and extra-cellular *Rickettsiae* in both tunica and peritoneum; *Rickettsiae* were seen both intra- and extra-cellularly in the spleen and in the liver; they were also present in certain elongated cells in the brain smears, possibly from the pia, and were found in small numbers in the blood.

In another animal similarly treated, except that two injections of benzol were given, the organisms were similarly distributed, except that while tunica and peritoneum were less heavily infected, the liver contained great numbers grouped in clumps and there were organisms extra-cellularly in the blood and—in one field—crowded in a mononuclear cell which, being found in the blood, might well be a desquamated capillary endothelial cell.

In another rat in which several benzol injections had been given, a similar result was obtained and the rat died on the fifth day after inoculation. The death of this animal, judging from the distribution of *Rickettsiae*, might well be regarded as having been due to these organisms, in which case we may have succeeded in possibly converting typhus into a fatal infection for rats. It is impossible, however, to be sure of this, since the death of the animal might have been due to benzol or to a combination of the two causes.

Material for sections of all the organs was taken, and sections will be studied for the purpose of differentially localizing the organisms in the various cells. We would include these studies in the present report were it not for the fact that our differential stain has not yet been successfully adapted to tissue staining, and Giemsa is apt to give such poor contrasts that organisms as small as the *Rickettsiae* are likely to be visible only in certain cell groups which may not represent the total distribution.

Figs. 2 to 7 are drawings and photographs made directly from slides of smears from two rats, showing the numbers and distribution of the *Rickettsiae*.

Experiment with the Inoculation of Washed Rickettsiae

The large numbers of extra-cellular *Rickettsiae* or Mooser bodies in the peritoneum of two of these rats gave us, we thought, an opportunity to carry out a crucial etiological experiment by washing the peritoneal exudate in Locke's solution and infecting guinea pigs with suspensions of *Rickettsiae* reasonably free from both blood plasma and cellular material.

Experiment I.—The peritoneum was washed with Locke's solution instilled through an incision in the peritoneal wall, about 15 cc. of Locke's solution being used. This material was centrifuged at a low speed for 5 minutes to remove most of the cells, the supernatant fluid again centrifuged for 5 minutes more to further remove cells, and then allowed to settle in the ice chest for a little less than two hours. Again at this time, the supernatant fluid was taken off and centrifuged at high speed for 1 hour. The supernatant fluid was then removed and the hardly visible sediment emulsified in several cubic centimeters of fresh Locke's solution. Two slides made from this suspension showed a not inconsiderable number of *Rickettsiae*, with only six cells that could be found by what we believe to be complete inspection of the two drops which had been allowed to dry on the slides. Two guinea pigs were injected subcutaneously with this material. Charts 1 and 2 made of these animals show only late and moderate rises in temperature, which are nevertheless suggestive of the typhus febrile movements often seen in subcutaneously inoculated Mexican typhus animals. These animals were intraperitoneally reinoculated with Mexican typhus blood on the 26th day, after the temperature had been normal for several days, together with a control. They remained normal, while the control came down typically, with a temperature of 104.2° and scrotal swelling on the sixth day, and on the seventh day a temperature of 106° and typical *Rickettsiae* in the tunica.

Experiment II.—Since we believed that in the first experiment attenuation of the *Rickettsiae* might have resulted from the length of time consumed in the washing, the exudate similarly taken from a second rat was centrifuged at low speed for 5 minutes, then the supernatant fluid from this centrifuged at high speed for 30 minutes. The sediment was again taken up in 15 cc. of Locke's solution, and again centrifuged at high speed for 30 minutes. This sediment was injected subcutaneously over the abdomen in Guinea pig 3. We specify the abdominal location of the subcutaneous injection because the subsequent testicular swelling in this animal might have been due to the escape of some of the injected fluid into the peritoneum. Our purpose in subcutaneously injecting was to produce, if possible, a European type of the disease, which would have been more convincing than a mere reproduction of the typical Mexican disease with the relatively pure *Rickettsiae* inoculum. As the chart of Guinea pig 3 shows, there was a typical rise of temperature, exceeding 104° on the twelfth day and almost reaching 105° on the

G.P.*1

May 19, 1930

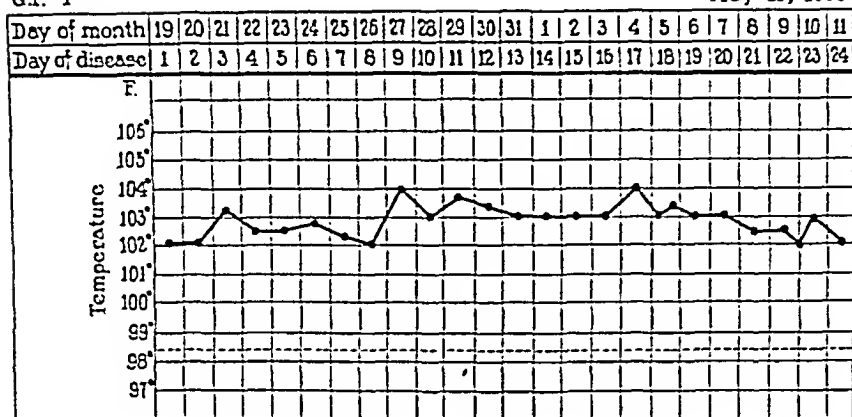


CHART 1

G.P.*2

May 19, 1930

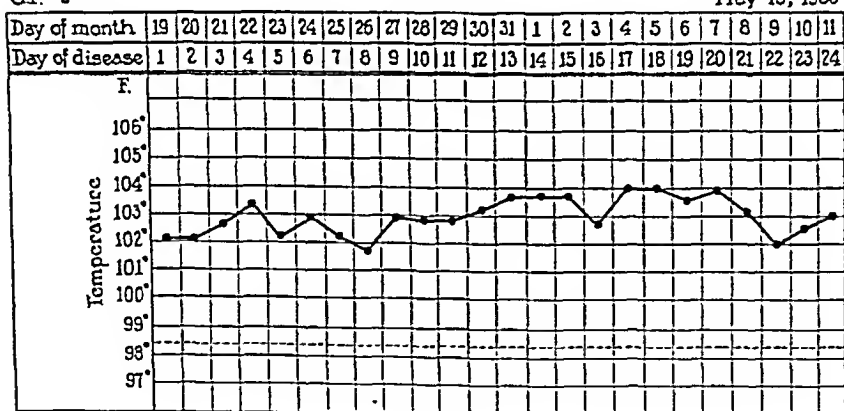


CHART 2

CHARTS 1 AND 2. The above charts are those of the two guinea pigs subcutaneously injected with thoroughly washed *Rickettsia* material in which microscopic examination showed many *Rickettsiae* and very few cells. The procedure took about 4 hours during which the material was about $1\frac{1}{2}$ hours in the ice box. It is not unlikely that the *Rickettsiae* were attenuated by this procedure, which may account for the unconvincing fever curves. These animals, however, were re-inoculated with typhus blood on the 26th day and showed no reaction whatever, while the control came down on the 7th day with a temperature of 104.2° and 106° on the 8th day with marked swelling. The washed *Rickettsiae*, therefore, immunized against the typhus blood, which indicates identity of virus and *Rickettsiae*.

thirteenth, a condition, as far as fever curve is concerned, more analogous to the European than to the Mexican type. However, on the fourteenth day scrotal swelling appeared, the animal was killed, and *Rickettsiae* were found in the tunica.

Although it is more difficult in Mexican typhus fever to find brain lesions and although this animal was killed rather early in the disease for the demonstration of these pathological changes, the brain of this guinea pig was removed, sectioned and stained by Giemsa. There was a certain amount of proliferation of vascular endothelium and a number of lesions were found which we regarded as suggestive of the typhus ones, and two of which Dr. Wolbach—from a greater experience with the pathology of this disease—tells us he would accept as typhus lesions.

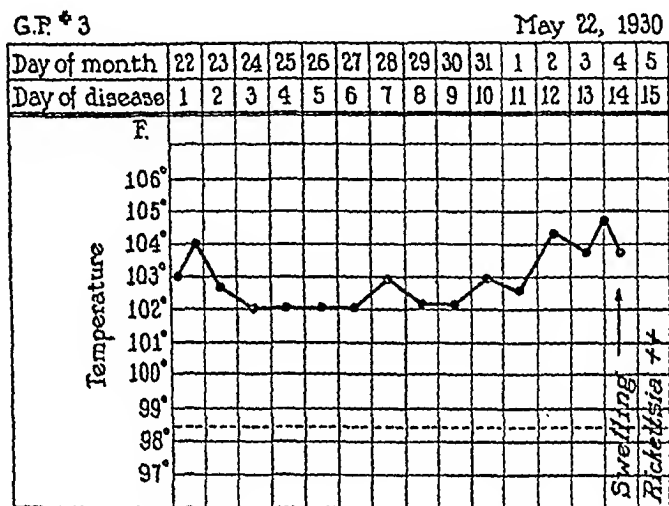


CHART 3. Temperature chart of Guinea pig 3, inoculated with washed *Rickettsiae* as described in Experiment II.

These experiments will have to be elaborated of course and repeated, but we publish them because we can see no obvious source of error, and we believe that we have reproduced in guinea pigs a disease which is clinically similar to Mexican typhus fever, including scrotal swelling and *Rickettsia* bodies, with material in which there might have been a very few endothelial cells from the peritoneum, but in which there was certainly not enough blood plasma to cause infection and which consisted, as closely as it is possible to accomplish this at the present time, of washed *Rickettsiae* or Mooser bodies.

DISCUSSION

By the use of benzol preliminary to inoculation with tunica material of guinea pigs, we have succeeded in a number of rats in producing a

massive invasion of *Rickettsiae* or Mooser bodies, with large numbers in the tunica and peritoneum and considerable numbers in the spleen, liver, pia mater and in the blood itself. The manner of action of the benzol is as yet undetermined, but we believe that it depresses resistance by the diminution of phagocytosis by the polynuclear neutrophils, which—from direct observation—we believe to be a powerful factor in the defence of the body against typhus fever. We have not yet succeeded in working out the quantitative and time factors by which these results can be achieved with regularity, but we have had perfect success in three instances and partial success in several others.

The benzol experiment, leading to a greater concentration of *Rickettsiae* in the blood, has made possible the bedbug feeding experiments described in our typhus Study III.

In obtaining large amounts of *Rickettsiae*, many of which were extracellular, in the peritoneum, we were enabled to carry out guinea pig inoculation experiments with washed *Rickettsiae*, free of serum and almost free of cells.

Two guinea pigs subcutaneously inoculated with such washed material, in which the process of washing had consumed about 4 hours, including over 90 minutes settling in the ice chest, presented atypical but—considering possible attenuation and the subcutaneous method of inoculation—probably true typhus reactions. This supposition is corroborated by the fact that these animals subsequently showed no reaction whatever to an intraperitoneal injection of Mexican typhus blood to which the control reacted typically in 7 days, with swelling and a temperature reaching 106°F. In a preceding paper we have presented evidence which suggests that guinea pigs can be actively immunized with formalinized tunica material. In such experiments, so far as our limited experience has gone, it was necessary to allow about a month to elapse between the last vaccine injection and the inoculation with living virus. In guinea pigs which have passed through a typhus infection immunity is established as soon as the temperature returns to normal. Since this was the case in these two animals, we believe that they had recovered from an attack of typhus conveyed by washed living *Rickettsiae*. In either case, the evidence is definite that the injected, washed *Rickettsiae* conferred typhus immunity.

The importance of this experiment lies in the fact that the subcutaneous injection of washed *Rickettsiae* from a Mexican typhus fever strain, free of plasma and almost entirely free of cells, produced a mild febrile reaction suggestive of, although not entirely characteristic of typhus fever. The mildness of the fever curve we attribute to the partial attenuation of the organisms in the course of washing. The absence of scrotal reaction was due to the subcutaneous manner of injection and renders the significance of the experiment rather greater than less. The fact that this inoculation with presumably pure *Rickettsiae* material protected these animals against an amount of virus which produced an unusually violent reaction in a control animal, with temperature, typical scrotal swelling and *Rickettsiae*, presents a train of evidence which in our opinion is incompatible with any interpretation other than that the *Rickettsiae* constitute the virus of Mexican typhus fever.

Another guinea pig, No. 3 (see Chart 2), after subcutaneous inoculation with washed *Rickettsiae*—a few of which probably filtered through to the peritoneum, since the injection was over the abdomen—developed a typical temperature curve with scrotal swelling and *Rickettsiae* in the tunica. This animal was killed on the thirteenth day, and though this was too soon to show well-developed brain lesions, a few lesions were found, which were histologically consistent with those described as characteristic of typhus by Otto and Dietrich, by Spielmeyer and by Wolbach.

CONCLUSIONS

We have adduced strong evidence in favor of identifying the virus of Mexican typhus fever with the *Rickettsiae*, or Mooser bodies, observed in the tunica lesions of Mexican typhus guinea pigs. Were it not for the possible presence of a few remaining cell fragments in the washings we would consider this evidence crucial proof.

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EXPLANATION OF PLATES

PLATE 32

FIG. 1. Drawing of phagocytosis of *Rickettsiae pr.* (Mooser bodies) in polynuclear leucocytes. Peritoneal exudate.

FIG. 2. Distribution of *Rickettsiae* in peritoneal scrapings.

PLATE 33

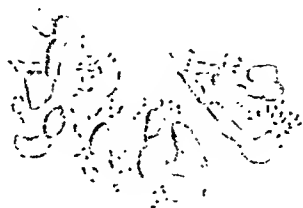
FIGS. 3 and 4. Photographs of peritoneal exudate in benzol, Rat IA, with cells crowded with *Rickettsiae* and extra-cellular organisms. (Kindly taken for us by Dr. Wolbach.)

PLATE 34

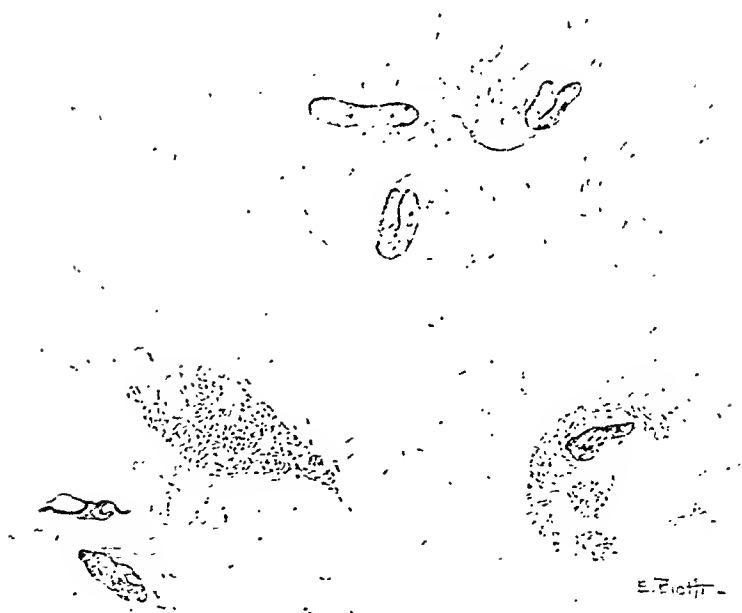
FIG. 5. *Rickettsiae* in liver, Rat IA.

FIG. 6. *Rickettsiae* in blood, Rat IA.

FIG. 7. *Rickettsiae* in elongated cells of pia from brain smears.



1



2







5



6



7

STUDIES ON TYPHUS FEVER

III. STUDIES OF LICE AND BEDBUGS (*CIMEX LECTULARIUS*) WITH MEXICAN TYPHUS FEVER VIRUS

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I

The Transmission of Mexican Typhus Fever through Lice Inoculated with Guinea Pig Organ Emulsions

One of the difficulties in the acceptance of Mooser bodies or *Rickettsia prowazeki* as the cause of typhus fever has been the fact that these organisms have not been found in the organs or blood of infected animals with which the disease can be transmitted. From titrations reported in a previous paper (1) it was quite obvious that inability to find the organisms in blood or tissues might well be due to their sparseness and the difficulty of recognizing them with certainty when isolated organisms are present in protein suspensions.

It would be strong evidence in favor of the presence of the *Rickettsiae* in the virulent organs not as incidental contaminations but as true etiological agents, if lice could be infected with such organ materials and from them guinea pigs inoculated, the disease produced and typical tunica *Rickettsia* lesions observed.

The actual injection of lice per rectum by the Weigl method is relatively easy, but it is not so easy to keep the lice alive after the injections, since guinea pig plasma and some of the guinea pig tissues seem to exert a toxic action to which lice succumb, although daily feedings on immune subjects are practiced. In consequence, out of seven experiments, only the seventh was successful, but there could be no possible error in this, the negative results being obviously attributable to technical difficulties.

The following brief description of all our experiments is given because some of the negative ones, we believe, have considerable value.

Experiments on Lice Transmission of Rickettsiae from Tissue

1. Feb. 8, 1930. Tunica of an infected guinea pig, containing many *Rickettsiae*, injected intrarectally into 20 lice. After 10 days' feeding, *Rickettsiae*, were found in the intestinal endothelium of a number of these insects. Guinea pig inoculation was not carried out with these lice.

2. Feb. 18. Brain suspension injected as above in 20 lice. Only 6 of these survived the 10 days' feeding. No *Rickettsiae* found, and no guinea pig injections done.

3. Feb. 27. 22 lice injected with virulent guinea pig plasma and human cells from defibrinated blood. Only 3 remained alive after 10 days, though regularly fed. No *Rickettsiae* found in these. The guinea pig control injected with the original plasma came down typically, with *Rickettsiae* in the tunica.

4. Mar. 19. 50 lice injected with human red cells which had been in contact with infectious guinea pig plasma and centrifuged. This was done on the basis of an experiment published in a preceding paper (1) in which it was shown that the typhus virus adhered tightly to the red cells after the exposure of normal cells to infectious plasma. The purpose of this was to eliminate the toxicity of the plasma. Only 2 of the lice lived 10 days. No *Rickettsiae* were found. Control guinea pig came down typically with *Rickettsiae* in the tunica.

5. Mar. 27. 42 lice injected with emulsion of suprarenal tissue from an infected guinea pig plus normal human red cells. After 10 days only 15 lice alive. No *Rickettsiae* found in smears and injected guinea pig was negative. The control guinea pig injected with the original material was positive, with *Rickettsiae* in the tunica.

6. Apr. 25. 25 lice injected with brain tissue. Of these only 16 survived 10 days. No *Rickettsiae* found in the lice examined. The guinea pig injected with them remained negative and the guinea pig injected with the original material remained negative as well.

7. May 8. 43 lice injected with a mixture of suprarenal and spleen tissue from an infectious guinea pig. 38 of these survived after 10 days' feeding. Since it is quite impossible to examine all the lice for *Rickettsiae* without ruining the material for injection, the ground suspension of a mixture of all the intestinal tracts was examined, but no *Rickettsiae* found. Two guinea pigs were injected with this suspension. One had a temperature of 105° with marked testicular swelling, on the 9th day and plentiful *Rickettsiae* in tunica smears (Chart 1). The other had fever on the 18th day. Temperature 105° and swelling on the 19th. No *Rickettsia* examination was made on this animal.

From these experiments it may be concluded that when the organs of a Mexican typhus guinea pig, during the infectious stage in which

morphologically no *Rickettsiae* can be found, are injected into lice by the Weigl method and these lice are then kept alive for 10 days by feeding on an immune human being, the *Rickettsiae* undoubtedly present in these organisms in small number survive and the intestines of these lice injected into a guinea pig will reproduce the original picture of the disease, including the typical *Rickettsia* lesions in the tunica vaginalis.

In the transmission of Mexican typhus from guinea pig to guinea pig with blood and organ emulsions by intraperitoneal injection, *Rick-*

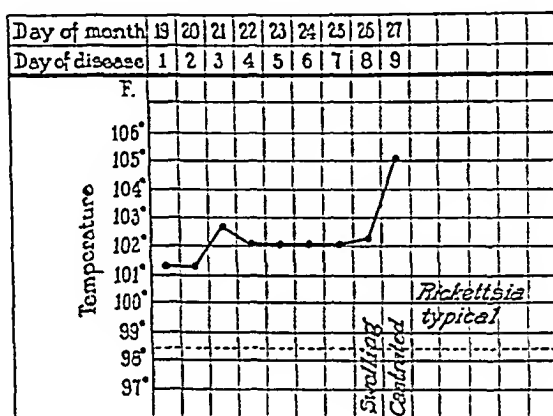


CHART 1. The above guinea pig was injected intraperitoneally on May 19 with an emulsion of lice that had been rectally injected with suprarenal and spleen tissue suspensions on May 8 and fed daily on an immune human subject in the interval.

ettsiae keep appearing in the tunica, and this alone would tend to indicate that these organisms were present in the organs without further demonstration. However, it may be—as it often has been argued—that possibly these *Rickettsiae* did not represent the actual virus but were incidental contaminations carried along with the virus.

Experiments like the successful one (No. 7) cited tend to indicate that the *Rickettsiae* present in the infectious organs can, like typhus virus, be carried for 10 days in infected lice and still be capable, after as many as eight to twenty normal feedings, of infecting guinea pigs in a typical manner. While this is not by itself conclusive proof of the identity of the Mooser bodies and the typhus virus, it adds another

link to the chain of favorable evidence, corroborating experiments like those of Mooser and Dummer (2) who found *Rickettsiae* in lice fed on Mexican typhus monkeys.

II

Experiments with Cimex lectularius

In the studies of Pinkerton in the Harvard Department of Pathology, as well as in our own studies and those of Maxcy, Dwyer and others, cross immunization and similarity of clinical and pathological findings in guinea pigs have identified the Mexican variety of typhus with the endemic typhus of the United States, which has been studied by Maxcy (3). Mooser has recently demonstrated the possibility of louse transmission with this disease, and undoubtedly such transmission can and does take place. On the other hand, the epidemiological studies of Maxcy indicate that, as he expresses it "There may be some mode other than direct transmission from man to man by means of the bite of a louse." In consequence, we determined to undertake the investigation of other insect possibilities in the transmission of Mexican typhus, and chose, as one of the most widely distributed and common, the *Cimex lectularius*, or bedbug.

Bedbugs collected from a non-typhus source—namely, in Boston—were kept in sealed Petri dishes with filter paper in the laboratory and first studied by dissection and staining of organ smears with the methylene blue-safranin stain and with Giemsa. The normal *Rickettsiae* of the *Cimex* have been thoroughly described by Hertig and Wolbach (4), whose studies aided us considerably in a preliminary survey of the organs of these insects. Although morphological differentiations are not particularly reliable in determining differences between virulent and non-virulent *Rickettsiae*, yet as a guide to work of the kind we are describing, the illustrations and descriptions of the writers named, compared with our own observation of Mooser bodies or *Rickettsiae prowazeki* have given us some confidence in the belief that tentative differentiations, while not conclusive, are still useful. We mention this because later in the experiment to be described we believe that we saw the Mooser type of *Rickettsiae* in considerable numbers in infected bedbugs.

Early feeding experiments with bedbugs having been negative, it was thought desirable to determine the amount of blood which a bedbug can ingest, since we know that the blood of guinea pigs, even at the most favorable time for reinoculation, is not infectious in amounts smaller than 0.05 cc., and rarely infectious in amounts lower than 0.1 cc.

2 adult bedbugs which had been starved for a number of days were weighed. The weight of the bedbugs before feeding was 0.012 gm. These 2 insects were then allowed to feed on an infected typhus rat and not removed until they had had their fill. They were then again weighed in the same balanced watch glasses and were found to weigh 0.0305 gm.

Subtracting the weight of the 2 bedbugs before feeding from their weight after feeding, and dividing this by 2, we obtain, as an approximate amount of blood taken up by each bedbug, 0.0093 gm., or slightly over 9 mg. of blood. Since it would then take approximately 10 bedbugs to take up a minimum dose for a guinea pig, we decided to carry out succeeding experiments by inoculating bedbugs with tunica material by a method analogous to the Weigl technique used in lice. This procedure is not very difficult, but the inoculated material passes into the coelom. In spite of this, however, the bedbugs survived.

Experiment on Injection of Bedbugs

Tunica material from Guinea pig 1, showing *Rickettsia prowazeki* or Mooser bodies on the fifth day after inoculation, was emulsified and injected with a very fine capillary pipette through the rectum into the coelom of 16 bedbugs. During the next 10 days they were fed three times on a normal guinea pig. This animal showed a slight fever for a day, but transfer from it did not produce typhus fever. 10 days after inoculation these bedbugs were killed, washed in 98 per cent alcohol and smears made from the gut and other organs. These smears, stained by the methylene blue-safranin method, showed—in one *Cimex*—bodies resembling the typhus *Rickettsiae*. The organs of these bedbugs were emulsified and injected into Guinea pigs 2 and 3. Both these animals showed rises of temperature and typical swelling. Guinea pig 3 was castrated on the sixth day of the disease and the second day of swelling. Plentiful *Rickettsiae* were found in tunica smears, and inoculation was done with this material into Guinea pig 4. This animal showed a typical rise, with swelling and positive *Rickettsiae*, on the fourth day. Transfer was made from this to Guinea pig 6, which, in the third generation from the bedbug, showed scrotal swelling on the sixth day, but no temperature. This animal was kept for immunity test and inoculated on the sixteenth day with brain of the European typhus strain. It remained normal, although controls came down typically. Guinea pigs 2 and 4 were reinoculated with European typhus and found immune.

The original guinea pig, No. 3, which had been castrated on the sixth day after the disease, was killed on the eleventh day, when the temperature was still high, and carefully autopsied. There were no lesions of other disease in the organs. Cultures from the spleen were negative and material from the brain was injected into Guinea pig 5. This animal, on the seventh day after inoculation, had a temperature of 104° and swelling, and plentiful *Rickettsiae* were found in the tunica after castration on this day. On the thirteenth day this guinea pig was autopsied and typical brain lesions were found. These have been verified for us by Dr.

Wolbach, to whom we are grateful for his helpful interest in these experiments. The results of this experiment are tabulated in the accompanying diagram.

Guinea Pig 1. April 18

Showed numerous *Rickettsiae* in tunica. Tunica emulsion injected into 16 bedbugs

April 28. Bedbugs dissected. Suspicious *Rickettsia prowazeki* seen in smears. Emulsion of organs intraperitoneally injected into

Guinea Pig 2

Temperature 104° and swelling on 7th day. Reinoculated with European typhus and found immune

Guinea Pig 3

May 3. Temperature 105° and swelling. Castrated. *Rickettsiae* plentiful. Tunica injected into

Guinea Pig 4

May 6. Castrated. *Rickettsiae* present. Reinoculated with European typhus and found immune. Injected into

Guinea Pig 6

No temperature but swelling on 6th day. Reinoculated with European strain on May 20. Immune. Controls typical on May 28

May 7. Killed. Brain injected into

Guinea Pig 5

Swelling and temperature 104° on 7th day. Castrated. *Rickettsiae* plentiful. Killed on 13th day. Brain sections show typical early lesions

Another experiment, similar to the above, was carried out with 25 bedbugs injected on Apr. 30. While they were being fed on the same normal guinea pig, on four separate occasions during an interval of 10 days, the feces were rubbed into the skin of this animal, but without result. Inoculation after 10 days of the organs of these bedbugs into a guinea pig resulted in typical disease with swelling and *Rickettsiae*. The disease was carried on from this animal through three generations.

Inoculation of Bedbugs by Feeding

After we had had the success in producing considerable accumulations of *Rickettsiae* in organs and in the blood in benzolized rats, as described in another paper, we thought it worth while to resume feeding experiments.

On May 16, 25 bedbugs were fed on benzol Rat IA on the third day after infection. On May 17, 10 of these bedbugs were again fed on a similar rat, IIB,

72 hours after infection. On May 18, 17 of the bedbugs were again fed on Rat IIB. On May 20 and 24, the surviving bedbugs were fed on a normal guinea pig. On May 25, the 20 surviving bedbugs were washed in 95 per cent alcohol, the intestines emulsified and injected into a guinea pig. Smears of these organs showed intra-cellular *Rickettsiae* suggestively but not conclusively similar to the Mooser bodies. This guinea pig, as shown in Chart 2, developed a typical temperature with swelling on the fourteenth day of disease and typical intra- and extra-cellular *Rickettsiae* were found in tunica smears.

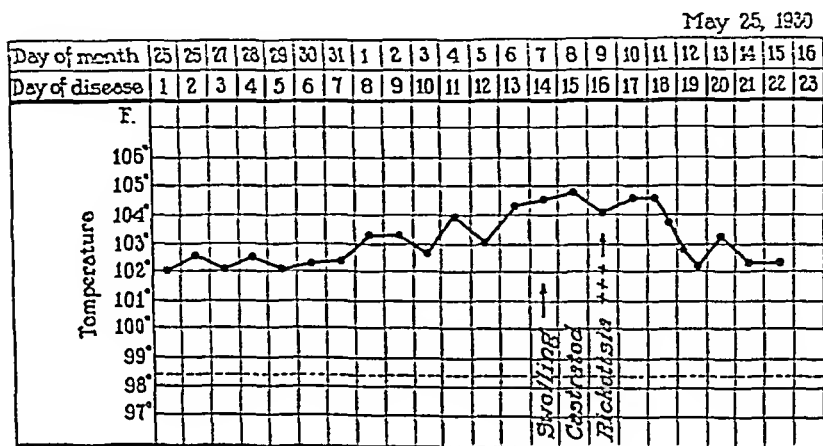


CHART 2. Chart of guinea pig inoculated with the organs of bedbugs which had fed upon benzolized typhus rats on the 9th, 7th and 6th days before the inoculation was done, and kept alive by feeding on a normal guinea pig in the interval. This guinea pig showed typical *Rickettsiae*, and was found immune to typhus on subsequent inoculation.

SUMMARY

Our experiments have shown that the Mooser bodies or *Rickettsiae* derived from guinea pigs with Mexican typhus fever can survive in bedbugs after intra-coelomic injection for 10 days, remaining capable of infection. We have also succeeded in similarly infecting bedbugs by allowing them to feed on benzolized rats in whose blood *Rickettsiae* had been shown to be present. Injection of the organs of such bedbugs 5 days after the last, 9 days after the first infectious feeding into guinea pigs produced typical Mexican typhus fever.

Some of the guinea pigs infected with such bedbug organs and pass-

ing through a typical typhus proved to be immune to subsequent inoculation with the European disease.

Attempts to infect normal guinea pigs by allowing infected bedbugs to feed on them or by rubbing the feces into the uninjured skin have, so far, been unsuccessful.

We have not, therefore, completed the cycle proving that bedbugs can transmit the disease, but we have shown that this is a possibility when dealing with man, obviously more susceptible to the disease than any of our experimental animals.

The ease with which the *Rickettsiae* seem to survive in the bedbugs suggests the desirability of investigating other common insects for a similar capacity of harboring the typhus *Rickettsiae*—experiments which we have not yet had the time to carry out.

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EXPERIMENTAL FIBROUS OSTEODYSTROPHY (OSTITIS FIBROSA) IN HYPERPARATHYROID DOGS

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PLATES 35 AND 36

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Ostitis fibrosa has been reported to occur spontaneously in animals other than man. Unsuccessful attempts have also been made to reproduce this condition by various experimental means. We have now produced the changes of true generalized ostitis fibrosa in guinea pigs and dogs (1-4). The experimental part of the present report describes in detail our results with dogs.

Spontaneous Occurrence of Ostitis Fibrosa in Animals

The bone changes of spontaneous ostitis fibrosa resemble in many ways, but frequently do not duplicate those observed in the fibrous osteodystrophies of man.

With regard to the appearance of osteoid tissue in the lesions observed in the spontaneous fibrous bone diseases of animals, Ingier (5) and Pick (6) stated that such lesions can be called ostitis fibrosa, even in the absence of osteoid tissue.

Peculiar to some animals with fibrosed bones is the common development of obstructions of the nasal and oral cavities, as these are encroached upon by thickening of their walls. As a result such animals may have sniffling respiration. However, fibrous bone lesions exist in animals not afflicted with sniffling respiration. Christeller (7), Rehn (8), Hintze (9), Busolt (10) and Ingier (5) described swine, goats, cattle and horses whose bones showed either true generalized ostitis fibrosa, or a local form of true ostitis fibrosa. In most instances only skulls were studied, but in a small number of cases complete examination of the skeleton was made. In these cases generalized ostitis fibrosa was found.

Koch (11) and Christeller (7) described spontaneous ostitis fibrosa in monkeys, Koch pointing out that this condition had been previously confused with rickets. White (12) detailed three cases of ostitis deformans in monkeys. Her cases were typical examples, both clinically and pathologically, of Paget's disease.

Dogs are also subject to spontaneous ostitis fibrosa. Christeller described a dog whose bones were thin and porotic. The long tubular bones showed curva-

ture and diminished longitudinal growth. In these bones the lesions were most prominent in the upper and lower portions of the diaphysis, where there was fibrous replacement of the marrow, transformation of the existing bone, new bone formation, bone resorption with numerous Howship's lacunae and osteoclasts. The haversian canals of the cortex were widened and invaded by fibrous tissue. The epiphyseal cartilage plates were present and relatively normal endochondral ossification was still in progress. The entire skeleton was involved, but the ribs showed relatively the greatest alterations.

Schmey (13) and Pick (14) described an atrophic deforming generalized bone disease in senile dogs with a predominance of osteoporosis, but with little fibrosis. Hager (15) described a case of *ostitis fibrosa* in a dog whose upper and lower jaws were tremendously enlarged. Weber's (16) dog, 8½ months of age, had spontaneous fibrous osteodystrophy with thickened porotic bones, cysts and giant-cell tumor formations. The jaw of his dog was also enlarged and cystic. The parathyroids were normal, and calcium metastases were not observed.

Attempts at Experimental Production of Ostitis Fibrosa

Many attempts have been made to reproduce experimentally all or some of the lesions of *ostitis fibrosa*—that is, cysts, hemorrhages, giant-cell tumors, marrow fibrosis, bone resorption and osteoid tissue.

Traumatization of the marrow has been extensively tried with the view of producing bone cysts or local *ostitis fibrosa*, but Lexer (17), Lotsch (18), and Nissen (19) have shown conclusively that injury, and the resultant hemorrhage into the marrow, do not lead to local cyst formation. Contrary to the opinion of Konjetzny (20) and Pommer (21), it seems logical to conclude that before an intramedullary hemorrhage results in local cyst formation there must be a generalized or an extensive local disease of the bone. The injection of fluids or the introduction of foreign bodies into the marrow cavity, injury of the bone or marrow by X-ray, by radium, by toxins developed in parabiotic animals, or by toxins of injected bacteria have led neither to true generalized *ostitis fibrosa* nor to true local *ostitis fibrosa*. The scarring, the marrow degeneration, or the cyst formation that may result from such treatment are only local reactive manifestations, having nothing to do with genuine *ostitis fibrosa*.

One-sided diets were among the first means employed in attempts at the experimental production of dystrophic bone diseases in dogs. Calcium deficiency was generally the underlying principle of the treatment. In most of the experiments the dogs received horse meat, fat or carbohydrates, and distilled water. The lesions produced in the various experiments were quite uniform. The older reports lacked detailed histological descriptions (22), and designated the changes produced as rickets or a ricket-like disease. Dibbelt (23) did not make a diagnosis.

It remained for Korssakoff (24) to point out that the condition produced in dogs on such a diet was osteoporosis and not rickets. Miva and Stoeltzner (25) studied

in great detail the changes produced by this diet and came to the same conclusion. They described marked osteoporosis with fibrosis of the marrow, but indicated that lacunar resorption was not severe enough to establish the lesion as *ostitis fibrosa*.

On a calcium-poor diet we, like some of the authors mentioned, have produced osteoporosis in dogs. These were fed lean meat but were protected against vitamin deficiency. On the other hand, in dogs which we maintained for a long period in a state of chronic hyperparathyroidism—both on adequate and calcium-poor diets—we produced generalized *ostitis fibrosa*. The clinical course, the evolution of the bone lesions, the gross and microscopical appearances of the bone and marrow were quite different in the two groups of animals, and indicated the specificity of the parathyroid extract treatment as the cause of experimental *ostitis fibrosa*.

The Relation of the Parathyroid Glands to Bone Dystrophies

Askanazy (26) was the first to suggest the etiological significance of a parathyroid adenoma found by him in a case of *ostitis fibrosa*. After his finding, many other instances of parathyroid enlargement were reported in association with *ostitis fibrosa deformans*, *ostitis fibrosa cystica*, osteomalacia, and rickets. Parathyroid enlargement has also been noted in rats suffering from experimental rickets.

Recently Barr and Bulger (27) summarized from the literature the cases with parathyroid tumors. They added 29 cases to those previously collected by Hoffheinz (28), making a total of 74 cases with pathologically enlarged parathyroid glands. These included both malignant and benign tumors, as well as enlargements due to simple hyperplasia. Authentic malignant growths of the parathyroids are extremely rare, and it is significant that none of them have been associated with evidence of functional derangement of the parathyroid glands as reflected in bone lesions. The majority of enlarged parathyroids reported are probably due to functional hyperplasia. The association of many diseases of bones with parathyroid hyperplasia or with benign tumor of the parathyroid glands is striking. Barr and Bulger state that 60 per cent of all cases of parathyroid enlargement had evidence of bone lesions. Clinically the most frequent as well as the most striking association is with the generalized form of *ostitis fibrosa cystica*—that is, the so-called v. Recklinghausen's disease.

The consensus of opinion until recently was that the parathyroid enlargement observed in association with these bone diseases was of a secondary nature, and appeared as a result of a compensatory hypertrophy due to the bone deficiency. In 1926 Mandl (29) brought clinical evidence to dispute this view. He removed a parathyroid tumor in a case of *ostitis fibrosa cystica* and reported rapid clinical

improvement of his patient, with decrease of urinary calcium excretion. He was the first to suggest, on the basis of his tests, that the skeletal changes in clinical cases were caused by a disturbed parathyroid function associated with the enlargement of the parathyroid gland.

Mandl's observations were soon confirmed and his conclusions widely accepted. A rapidly increasing series of reported cases supports the hypersecretion hypothesis. Gold (30), Barr, Bulger and Dixon (31), Barr and Bulger (27), Wilder (32), Boyd, Milgram and Stearns (33), Snapper (34), Richardson, Aub and Bauer (35), and Compère (36) reported cases showing a varying amount of improvement following extirpation of enlarged or even normal-sized parathyroids when hyperparathyroidism was diagnosed.

Hypercalcemia, and negative calcium balance when this has been demonstrated, may be attributed to the increased secretion of its active principle by the enlarged parathyroid gland. The case reported by Richardson, Aub and Bauer (35), by Bauer, Albright and Aub (37), by Hannon, Shorr, McClellan and DuBois (38), and by McClellan and Hannon (39) is important. In this case, which presented a clinical picture of hyperparathyroidism, no parathyroid tumor was found, and two normal appearing parathyroid glands were removed. The improvement in the condition of the patient was so marked that it indicated to these authors that hyperparathyroidism may result from hyperfunctioning of glands of normal size and appearance. The possibility exists that some undiscovered or abnormally active accessory parathyroid tissue may produce hyperparathyroidism in clinical cases where a tumor is not found after exploration.

A review of the symptoms in reported cases diagnosed as suffering from hyperparathyroidism, and of the clinical and chemical findings, reveals certain conditions common to all. Among these are: progressive muscular weakness, general lassitude and hypotonia; pain in and bowing of the lower extremities; resorption of the skeletal bones, frequently shown to be associated with hypercalcemia and with a negative mineral balance. Following operation the calcium balance, when this was studied, generally became positive. Fractures which occurred during the course of the disease healed very slowly (30, 31, 40) but soon after parathyroidectomy a fractured femur united firmly (34). Following removal of the parathyroid tumor, X-ray showed increased density of the bones in some of the cases (30, 31, 32, 34, 35). No positive evidence of healing of the bone cysts was included in the reported cases. Giant-cell tumors of the bones were noted in the cases of Wilder and of Barr, Bulger and Dixon, and these were found to be healed within a few months after removal of the parathyroid tumor.

Some of the reports cited above, available to us when our work began, indicated clearly that parathyroid adenomas or parathyroid hypersecretion were etiologically related to *ostitis fibrosa cystica*. We therefore felt that injections of parathyroid extract into experimental animals might produce bone lesions similar or analogous to

those found in clinical *ostitis fibrosa*, provided the animals could be maintained in a state of hyperparathyroidism without succumbing.

It is interesting to note that although a number of investigators injected parathormone into experimental animals for long periods, and although increased excretion of calcium at the expense of the bones is well established, no investigator has studied the bones of such animals histologically.

Experimental work with parathormone by Collip (41), Greenwald (42) and others had demonstrated that its injection led to hypercalcemia and to increased excretion of calcium and phosphorus, particularly in the urine; to lowered serum phosphorus and hypotonicity. Overdosage phenomena had also been described by Collip, Hueper (43) and Learner (44) in experimental animals (vomiting, diarrhea, hematuria, hemorrhagic gastro-enteritis, kidney impairment and hyperphosphatemia, and extensive metastatic calcification in the soft tissues, especially the kidneys, lungs and gastro-intestinal tract).

A careful study of the effects of daily injections of parathormone on rabbits, kittens and rats was made by Bauer, Aub and Albright (45). They were primarily interested in the question of whether the trabeculae serve as a reserve supply of calcium. After continued parathormone administration to rabbits for 91 days, during the major part of which they received 8 units daily, the trabeculae were reported as diminished in number, but no effect was noted on the cortex of the bone either in the gross examination or by X-ray. In growing kittens the administration of parathormone, on the average of 25.4 units daily for 56 days, did not result in any greater reduction of the trabeculae than observed in the course of normal bone growth. In the growing rat the administration of parathormone, 885 units over 110 days, resulted in diminution of the length of the bones and in an increase of the number of trabeculae. The serum calcium of the treated rabbits was higher than in the controls, while the serum calcium of the treated kittens was reported lower than in the controls, but there were no changes in the rats. They concluded, in spite of the fact that their own data do not seem to support their view, that bone trabeculae are easily depleted by prolonged administration of parathormone, and that these trabeculae serve as the source of readily available calcium.

Ingier (5), Christeller (7), Stenholm (46), Pick (6), Schmorl (47) and others emphasize the necessity of making histological studies of bone even in human cases, but especially in animal bone, before drawing conclusions regarding bone changes. Our own experience confirms

them. We have been frequently surprised at finding, upon histological examination, extensive resorption of cortical bone in our experimental animals, when the gross examination did not show anything obviously unusual. We believe that the results of Bauer, Aub and Albright, without the evidence of an histological examination, cannot be accepted as proving the absence of cortical resorption. Aub, Bauer and Albright based some of their conclusions on absence of hypercalcemia and on negative gross examinations. That these are not a proof of lack of parathormone effects is shown by our results in guinea pigs, which while receiving doses not large enough to produce hypercalcemia, still showed cortical bone resorption microscopically (1, 2, 48). In dogs as well (Nos. 3 and 4, see protocols), cortical resorption was produced on 2 units of parathormone daily for 20 days, although hypercalcemia was never observed.

Experimental Methods

We started with puppies 6 to 9 weeks of age, weighing 1 to 2 kg. The diet consisted of lean meat fed daily in amounts of about 15 per cent of body weight. Later, when active growth ceased, the amount fed was reduced to 10 per cent of body weight. These amounts corresponded actually to feeding *ad libitum*; the meat was frequently not consumed in full. Records of meat consumption were kept. The meat was supplemented daily with 1 cc. of cod-liver oil and at least 50 gm. of canned tomato. During various phases of the experiment known supplements of calcium were given in the form of calcium lactate by stomach tube, or calcium lactate and bone meal were added to the meat. The dogs were kept under carefully controlled hygienic conditions.

Serum calcium and phosphorus determinations were done at frequent intervals. The Clark-Collip modification of the Kramer-Tisdall method was employed in the calcium analyses, and the Benedict-Theis method in the phosphorus analyses. If the animal was receiving parathormone, the blood was generally examined about 18 hours after the last injection, the animals being fasted during that interval. The parathormone was injected subcutaneously in daily doses, which were increased as indicated by the course of the experiment.

The tissues and bones were fixed in neutral formalin or in Helly's fluid. The bones fixed in formalin were decalcified in Mueller's fluid plus 5 per cent glacial acetic acid. Those fixed in Helly's fluid were decalcified in 5 per cent nitric acid. When tissues were stained for calcium, they were fixed in 95 per cent alcohol and the v. Kossa method was used. Paraffin sections were always made. The details of these methods as employed in this laboratory have been described (49).

Experimental Results

After having gradually increased the daily dose of parathormone, we found that the dog could tolerate relatively large doses of the extract without showing hypercalcemia. We attribute the production of experimental *ostitis fibrosa* in dogs to the increased parathormone effects obtained by prolonged administration of relatively large doses of the extract. Otherwise the production in dogs of *ostitis fibrosa* would be difficult, because doses of parathyroid extract large enough to produce rapid resorption of the bone and injury to the marrow, lead in the dog to fatal hypercalcemia before there is much fibrous repair.

The parathormone was injected in a daily dose, at first usually of 2 units per kilogram, which was raised gradually in some dogs to as high as 5 units per kilogram towards the end of the experimental period of 5 to 6 months. Repeated small daily injections of parathormone at first usually raised the serum calcium to about 15 or 16 mg. per 100 cc. It was sometimes necessary to interrupt the parathormone treatment because of loss of appetite and other symptoms of overdosage. As treatment was continued, the serum calcium gradually fell to normal values or lower, and larger doses could be given with only a temporary elevation of serum calcium. This condition was produced most rapidly in one dog on a low calcium intake. In that dog (No. 8), serum calcium indeed dropped to as low as 7.8 mg. per 100 cc., and seemed to be associated with a tissue calcium lowered sufficiently to account for the tremors and tetany observed during the experiment (see protocol).

Immunity acquired as a result of previous parathormone treatment cannot be inferred from the normal serum calcium values in dogs treated for a long time with parathormone (Nos. 9 and 10), and from the hypocalcemia observed in one instance (No. 8). For when the calcium reserves were allowed to replenish after discontinuing parathormone for a short period, parathormone injections had their usual effect of raising the serum calcium (see protocols).

We produced, depending upon the dosage and the length of time under parathormone, all degrees of change from mild to severe bone resorption, and from slight fibrous replacement to degeneration of the marrow with hemorrhage, when the animals died from overdosage. Finally, we produced typical *ostitis fibrosa cystica* in several dogs. The most pronounced lesions were observed in three dogs that were

injected for 5 to 6 months. At the end of their experimental periods they were receiving 20 units daily.

One of the most marked clinical features in all these young growing dogs was the stunting of their growth, which was referable to a cessation of bone formation, and therefore of growth at the epiphyseal cartilage plates. After prolonged treatment the bones were deformed, and the forelegs showed lateral bowing. Such animals had pronounced hypotonicity of the muscles and developed plantigrade stance. In addition there was frequently torsion of the tubular bones on the long axis. The bone changes were generalized and lesions were found in the jaw bones, the skull, and all the long tubular bones studied. The ribs in the vicinity of the costochondral junctions offered the best material for study. In the long tubular bones, the fibrous changes were present only in the diaphysis and metaphysis. The epiphyses showed only a mild degree of simple resorption of the trabeculae and the marrow remained entirely free of fibrous changes. It is important to emphasize that none of our dogs had swellings at the epiphyseal cartilage plates, nor were there any indications of widened metaphyses or costochondral junctions.

In view of the frequent confusion in interpretation of the histological picture found in clinical or experimental bone dystrophies diagnosed as osteomalacia, osteoporosis and ostitis fibrosa, it is important to emphasize the criteria which must be satisfied by the lesion to which the term generalized ostitis fibrosa is applied. These are (a) resorption of the existing spongy and cortical bone, (b) invasion of the enlarged haversian spaces and of the marrow canal by fibrous tissue, and (c) the presence of Howship's lacunae, containing osteoclasts, on the walls of the haversian spaces, on the inner and outer surfaces of the compacta, and on the surfaces of the spongy trabeculae. New bone formation (osteoid tissue), as a substitute for the original lamellar bone, and cysts and hemorrhages in the marrow cavity, while frequently found, are in our opinion not essential for the diagnosis of ostitis fibrosa. Osteoid tissue appears as a result of the reparative process going on in bones subject to ostitis fibrosa, and may be absent, slight or extensive, depending on the functional need for reinforcement by osteoid repair.

Upon histological examination all the stated essential criteria of

ostitis fibrosa were found to be present in the dogs suffering from chronic hyperparathyroidism.

The fibrous bone changes were produced in dogs on a high or low calcium intake. In one dog (No. 7), receiving 650 mg. of calcium daily in the form of calcium lactate administered by stomach tube, considerable fibrous replacement of the bone was observed on a dose of 8 units of parathormone daily during the last 30 days of the parathormone treatment. The production of ostitis fibrosa was enhanced in the dogs by depleting their calcium stores more rapidly through the diminution of the calcium intake, which permitted giving larger doses of parathormone without danger of fatal hypercalcemia. When this condition is realized, an essential prerequisite exists for the production of ostitis fibrosa by parathyroid extract.

That the changes observed could not be attributed to calcium deficiency alone is evident from examination of bones of control dogs kept for months on a low calcium diet. These animals developed osteoporosis, which is histologically quite different from ostitis fibrosa cystica. Furthermore, by the use of parathormone, we have produced ostitis fibrosa in guinea pigs that were receiving a normal diet of adequate calcium content (1, 2).

PROTOCOLS

In the following protocols of 10 dogs on parathyroid treatment, only those data have been included which seemed necessary to a clear understanding of the means employed in the experimental production of ostitis fibrosa and of the results observed in the bones and tissues. The procedure had necessarily to be suited to each animal and was necessarily modified by developments. The central object was to preserve the life of the animal long enough to elicit the effects of experimental hyperparathyroidism on bone. A detailed presentation and discussion of the chemical data obtained will be published elsewhere, but sufficient data are given here to throw light on the clinical course of experimental hyperparathyroidism. The serum phosphorus data are given only when they indicate an exceptional condition. Some dogs died during the course of the experiments. With others, the experiments were terminated by administration of ether.

Dog 1.—Initial weight, 2.3 kg. Weight at the conclusion of the experiment, 11 days later, 2 kg. Calcium supplement, 600 mg. in the form of bone meal and calcium lactate. Four units of parathormone daily for 6 days and 8 units for 4 days. The dog ate all food until the 9th day, when complete anorexia appeared. The dog died on the morning of the 11th day. Serum calcium, 17.0 mg. per 100 cc. on the 10th day.

Autopsy showed loss of abdominal fat, involution of the thymus, and decrease of the external parathyroids to about one-half of the usual size. An hemorrhagic area was present in the left upper lobe of the lung; the kidneys were pale; the bones broke easily. Histological examination showed bone resorption, but the marrow changes formed the most striking feature. There was extensive necrosis of the marrow of all the bones examined. The marrow injury was most marked at the metaphyses and costochondral junctions. It was least pronounced or absent in the epiphyses. Fragmentation of the nuclei of the marrow cells was a prominent feature. The megakaryocytes were very badly degenerated and had entirely disappeared from some sections. Numerous mature neutrophilic and eosinophilic leucocytes appeared in the marrow. The erythrogenic cells were also degenerated. The connective tissue reticulum of the marrow was proliferated. There was diapedesis of red blood cells, particularly in the metaphysis, extensive dilatation of blood vessels, and considerable free marrow hemorrhage. Small collections of fibrin were observed in the hemorrhagic areas, and phagocytes also appeared in the marrow. The osteoclasts were large and numerous and for the most part not degenerated, indicating that they were reactive cells. In addition to the very extensive degeneration of the marrow, the osseous tissue of the ribs and tubular bones showed evidences of very rapid decalcification. The trabeculae and the walls of the haversian canals were nearly completely stripped of cells. The cartilage cores in the areas of endochondral ossification were either nearly bare or bare by the resorption of the previously deposited fiber-bone. New endochondral bone formation had ceased. This bone resorption was undoubtedly due mainly to vascular processes. Osteoclasts and Howship's lacunae were present, but mostly under the periosteum. Embedded and ground-disk cross-sections from the middle of the diaphyses of the long tubular bones showed enlargement of the haversian canals with Howship's lacunae containing osteoclasts. Examination of the soft tissues demonstrated extensive degeneration of the centers of the Malpighian corpuscles of the spleen, with congestion and leucocytic infiltration of the pulp; involution atrophy of the thymus; congestion of the lymph nodes; and congestion, leucocytic infiltration and pigmentation of the liver. The kidneys showed some calcium casts in the tubules. There was evidence of pneumonia, and the suprarenals were congested, with leucocytic infiltration. The tissues were not fixed or stained especially for calcium.

Summary.—This dog died of acute hyperparathyroidism. One of the most striking changes was in the bone marrow, which was ex-

tremely degenerated and hemorrhagic. The bones showed extensive decalcification. There had as yet been little marrow repair. The lymphoid tissue of the spleen was also degenerated.

Dog 2.—Initial weight, 1.65 kg. Weight at conclusion of experiment, 14 days later, 1.4 kg. No supplementary calcium was added to the diet. Four units of parathormone were given for 6 days, and 6 units for 3 days. On the 9th day the dog lost its appetite. On the 10th day it had bloody stools, and its serum calcium was 16.5 mg. per 100 cc. The dog died on the 14th day. Autopsy showed involution of the external parathyroids to about one-half the usual size and bilateral pneumonia and fibrinous pleurisy on the left side. Histological examination showed numerous degenerated kidney tubules containing calcium casts. Some glomeruli contained calcium deposits. The spleen was scarred, and showed evidence of active phagocytosis and leucocytic infiltration. The lungs contained patches of pneumonia and granules of deposited calcium. The bones were not studied. The parathyroids showed simple involution atrophy.

Summary.—This dog died of acute hyperparathyroidism complicated by bronchopneumonia. The soft tissues showed metastatic calcification.

Dog 3.—Initial weight, 1.95 kg. Weight at the termination of the experiment, 21 days later, 2.3 kg. Calcium supplement, 200 mg. daily, in the form of bone meal and calcium lactate. Injected 2 units of parathormone daily for 20 days. Serum calcium was 12.4, 11.7, and 12.8 mg. per 100 cc., respectively on the 11th, 18th and 21st days. The dog was killed to terminate the experiment. Autopsy showed a good state of nutrition. The organs and bones showed nothing unusual in the gross. The epiphyseal cartilage plates were thin. The thick cortex of the shafts of the long bones appeared compact in the gross. But when compared with a normal litter control, ground disks made from sections taken from the middle of the diaphysis of the long tubular bones showed definite microscopical evidence of resorption even of the apparently compact cortex. The Schwalbe and haversian canals were enlarged, but in stained sections no excessive connective tissue invasion of the canals was observed. In the stained sections of the long tubular bones no disturbance of endochondral ossification and no fibrous replacement of the marrow were found. The ribs, however, even with the small doses of parathormone received by this dog, showed degeneration of the blood-forming cells of the marrow, with destruction of the megakaryocytes which were being phagocytosed. There was fibrosis of the marrow, with the appearance of numerous leucocytes and eosinophiles. At the costochondral junctions rapid decalcification had taken place, and the more compact bone showed evidence of lacunar resorption with osteoclasts. The soft tissues showed nothing very unusual. Some kidney tubules contained calcium casts.

Summary.—Pronounced effects on the bone and marrow were observed even when parathormone was given for a short period in doses not sufficient to induce a hypercalcemia. The bone resorption was generalized and quite evident in the cortex of the diaphysis. The marrow changes were strikingly present in the ribs.

Dog 4.—Initial weight, 1.7 kg. Weight at the conclusion of the experiment, 21 days later, 1.95 kg. Calcium supplement, 600 mg. daily (bone meal and calcium lactate). Two units of parathormone were injected daily for 20 days. The serum calcium was 14.2, 12.4, 12.2, and 12.0 mg. per 100 cc., respectively, on the 11th, 12th, 18th and 21st days. The animal was killed to terminate the experiment when it was in excellent condition. Grossly neither the bones nor the soft tissues showed anything unusual. In ground disks of sections from the middle of the shafts of the long tubular bones resorption, as evidenced by enlarged vessel canals, was observed. Stained longitudinal sections of the long tubular bones showed some resorption of the compact and spongy bone, but connective tissue scarring was absent. The ribs, as in Dog 3, showed destruction of marrow cells, rapid decalcification at the costochondral junctions, osteoclasts and Howship's lacunae. There were calcium metastases in the kidneys. Otherwise the soft tissues showed no unusual features.

Summary.—In this dog, as in Dog 3, bone resorption and marrow fibrosis occurred after small doses of parathormone.

Dog 5.—Initial weight, 1.3 kg. Weight at conclusion of the experiment, 33 days later, 2.5 kg. Calcium supplement, 280 mg. daily for 5 days (in the form of bone meal), and 100 mg. (bone meal and calcium lactate) to the end of the experiment. The animal ate all its food until the 37th day, when it suddenly developed complete anorexia. The dog received 2 units of parathormone daily for the first 7 days, then 4 units daily for 17 days, 8 units daily for 4 days, 12 units daily for 6 days, and finally 16 units daily for 2 days. On the 37th day the parathormone was discontinued. At this time the dog was not obviously moribund. It was found dead on the 38th day. On the 12th day the serum calcium was 12.0 mg. 100 cc.; on the 28th, 11.6 mg.; and on the 34th day 11.0 mg. The blood obtained post-mortem showed a serum calcium of 13.6 mg. The dog probably died about 30 hours after the last injection. The urine obtained from the bladder at autopsy was acid (total titratable acidity equivalent to 660 cc. N/10 hydrochloric acid per liter). The autopsy showed that the dog was well nourished. There was considerable oedema of the subcutaneous tissues of the neck. The external parathyroids were reduced to from one-third to one-half their usual size. The lungs showed hemorrhagic and oedematous areas. The rest of the soft tissues presented nothing unusual in the gross. The bones were soft and brittle, and broke easily; the skull bones were easily cut with the scissors. Histological examination of the

bones showed rapid decalcification. The subepiphyseal cartilage plate trabeculae were reduced to cores of cartilage. New bone formation had ceased at the epiphyseal cartilage plates and costochondral junctions. The marrow was necrotic in places. Much of the cellular marrow had disappeared from the diaphysis. The marrow was fibrosed and contained leucocytes. The resorption of the bone was shown by the naked trabeculae and by the enlarged haversian canals. Numerous osteoclasts in Howship's lacunae were observed, but the hemorrhages and the vascular dilatations indicated that much of the resorption was vascular. While there was extensive resorption even in the shafts of the tubular bones, no osteoid tissue was observed in the marrow. The thymus was involuted. The lungs showed bronchitis and broncho-pneumonia and irregular collections of calcium were observed in the inflammatory exudate. The spleen was somewhat fibrotic and there was considerable necrosis of splenic cells. The parathyroids showed simple involutionary changes; the cells were to a great degree spindle-shaped.

Summary.—The dog undoubtedly died of hypercalcemic shock due to overdosage. It showed the acute effects of parathormone on the bone and marrow, superimposed upon the chronic effects of the previous treatment. A striking feature was the absence of osteoid tissue, which apparently does not appear until there is pronounced decalcification necessitating repair.

Dog 6.—Initial weight, 1.1 kg. The dog attained a weight of 2.1 kg. about 2 weeks before its death, which occurred 65 days after the beginning of the experiment. At autopsy it weighed 1.8 kg. The calcium supplement consisted of 280 mg. calcium in the form of bone meal for 5 days; of 100 mg. of calcium (bone meal and calcium lactate) for the next 36 days; it was increased to 1200 mg. for 5 days; no calcium was given for 2 days, and finally 650 mg. were given in the form of 50 cc. of a 10 per cent calcium lactate solution by stomach tube daily for 17 days. The dog received 2 units of parathormone for the first 7 days, 4 units for 5 days, the serum calcium rising to 14.8 mg. per 100 cc. on the 12th day of the experiment. Parathormone was discontinued for 7 days and was again begun on the 19th day of the experiment at 2 units daily. This dose was continued for 6 days, followed by 4 units daily for 4 days, 6 units daily for 6 days, and 10 units daily for 7 days. The serum calcium on the 27th, 33rd and 41st days was 12.2, 11.2 and 10.6 mg. per 100 cc., respectively. There was a progressive drop in spite of increased parathormone dosage, which was probably due to the fact that the dog was getting only about 100 mg. calcium daily during this period. The last serum calcium value was obtained just before the calcium supplement was increased to 1200 mg. daily on the 41st day. On the 43rd day, the dog still being on 10 units of parathormone daily, the serum calcium rose to 15.3 mg. per 100 cc. The parathormone was discontinued for 4 days. Treatment was resumed on the 47th day, with 6 units daily. The calcium intake was reduced

to 650 mg. by stomach tube. On the 51st day of the experiment serum calcium of 19.0 mg. per 100 cc. was found, and 18.5 mg. on the following day. On continuing the treatment, the serum calcium dropped to 12.6 mg. by the 58th day and on the 61st day it was 11.3 mg. The dog showed pronounced loss of appetite on the 57th day and left almost all of its food from the 62nd day on. It died on the 65th day. 2 hours before death the serum calcium was 15.8 mg. and the serum phosphorus 11.9 mg. per 100 cc. (The coincidence of these high values is a common observation in fatal hyperparathyroidism.)

At autopsy the external parathyroids seemed to be slightly smaller than usual. There were patches of pneumonia in the left lower, and right lower and middle lobes of the lung. The heart muscles were pale and oedematous. The kidneys were yellowish, the markings indistinct, and throughout the kidney white specks, apparently calcium deposits, were seen. There was an intense gastro-enteritis. The stomach wall was thickened to about five times normal. The stomach mucosa was extremely injected, and showed numerous mottled light-yellow areas, apparently containing calcium deposits. The injection of the intestines extended to the end of the ileum; the sigmoid and colon were free. The bones were softened, and broke easily. There were no swellings at the costochondral junctions.

The ribs on histological examination showed marrow fibrosis, but some lymphoid marrow was still present. The cortical bone was resorbed, and there were numerous Howship's lacunae and osteoclasts. Endochondral ossification was still in progress, the new formed trabeculae being lined by osteoblasts. Sections of the long tubular bones also showed marrow fibrosis and bone resorption, but to a lesser degree than the ribs. In ground-disk sections from the middle of the diaphysis enlarged vessel canals were found. The lungs were consolidated and calcium deposits were present in the inflammatory exudate. The stomach and intestines were congested, necrotic in places, and calcium deposits were found in the mucosa. The kidney tubules contained calcium casts. The parathyroid sections showed involutional atrophy of the parenchymatous cells.

Summary.—On a moderate calcium intake parathormone dosage was increased rapidly without adverse effects. When the calcium intake was increased, the serum calcium rose at first, but soon declined to normal values. However, the dog was apparently in a state of severe hyperparathyroidism for about 1 week before its death while it showed normal serum calcium values. There was terminal hypercalcemia and hyperphosphatemia. At autopsy the severe effects of acute hyperparathyroidism were observed in the soft tissues, while the bones showed the effects of chronic hyperparathyroidization. It is noteworthy that osteoid tissue formation was not prominent.

Dog 7.—Initial weight, 1.5 kg. Killed accidentally 106 days later, when it weighed 4.35 kg. The diet was supplemented with 1400 mg. of calcium in the form

of bone meal for the first 5 days. During the next 64 days 600 mg. of calcium (bone meal and calcium lactate) were added daily. Following that, for 37 days the dog received daily 50 cc. of 10 per cent calcium lactate by stomach tube (equivalent to 650 mg. of calcium), and was killed accidentally on the 106th day of the experiment by suffocation when the stomach tube was inserted in the trachea. Two units of parathormone were injected daily for the first 7 days, then 4 units for 17 days, 8 units for 4 days. The serum calcium values were 11.9 on the 12th day, and 11.3 on the 28th. Parathormone was increased to 12 units for 2 days, but was discontinued for the next 4 days because anorexia developed; no serum calcium determination was made. Before parathormone injections were resumed, serum calcium was 11.4 mg. per 100 cc. (on the 34th day). It rose to 15.6 mg. on the 39th day of the experiment, after parathormone had been given for 4 days (10 units daily). This was the first time hypercalcemia was observed in this dog, although signs of hyperparathyroidism had been present (anorexia). Parathormone was discontinued for 2 days, and then resumed, 8 units being given daily for 21 days until the 61st day of the experiment. The serum calcium was 12.2 mg. per 100 cc. on the 42nd day, 12.6 mg. on the 45th, 13.7 mg. on the 53rd, and 11.1 mg. on the 60th day of the experiment. Parathormone injections were increased to 14 units daily on the 62nd day. On the 63rd day the serum calcium rose to 14.5 mg. On the 67th day the serum calcium was 17.1 mg. but serum phosphorus was not raised. The animal refused food though he was active. Parathormone was discontinued for 2 days, and then resumed at 6 units daily. After 8 days it was increased to 8 units daily, which dose was continued from the 77th day to the 106th day. The serum calcium showed a regular drop from 11.5 to 9.8 between the 74th and 97th day of the experiment, in spite of the daily parathormone injections.

The animal at autopsy was well nourished. There was considerable abdominal fat. The soft tissues showed no gross pathological lesions. The dog was stunted in growth, the bones were short, and the cortex of the long tubular bones was thin. The ribs showed fibrosis of the marrow. The lymphoid marrow had been largely replaced by loose connective tissue containing numerous eosinophiles and a sprinkling of marrow cells. The blood vessels were dilated. Near the costochondral junctions and underneath the periosteum there were numerous osteoclasts with evidence of active osteoclastic resorption. A few small cysts were seen throughout the marrow. These were lined by flattened endothelioid cells and probably represented blood vessels dilated as a result of circulatory stasis. The haversian spaces of the compacta were enlarged, but in spite of the fibrosis of the marrow and the resorption of the bone most of the trabeculae were lined by osteoblasts and active endochondral ossification was in progress. There was metaplastic new-bone formation. Sections of the long tubular bones showed essentially the same changes as the ribs, but in somewhat lesser degree. The ground disks of sections from the diaphysis of the long tubular bones also showed resorption, the vessel canals being much enlarged. The parathyroid showed involutionary changes and metastatic calcification of the kidney tubules and stomach mucosa was found.

Summary.—On parathormone therapy with an intake of at least 600 mg. of calcium daily there was bone resorption and marrow fibrosis, but there was also new-bone formation. The bone was deposited on cartilaginous trabeculae in the course of endochondral ossification. Having succeeded in maintaining this dog for a longer period than No. 6, we obtained a more severe degree of bone resorption and marrow and bone fibrosis.

Dog 8.—Initial weight, 1.1 kg. Killed to terminate the experiment 168 days later, at which time it weighed 2.9 kg. No supplementary calcium was given except between the 52nd and 60th days, when the dog received 650 mg. of calcium daily in the form of calcium lactate by stomach tube.

This dog received at first 2 units of parathormone daily; from the 8th to the 24th day, 4 units daily; from the 25th to the 29th day, 6 units daily; from the 30th to the 34th day, 12 units daily; from the 35th to the 39th day, 16 units daily; from the 40th to the 48th day, 6 units daily, and from the 49th to the 51st day, 10 units daily. Between the 52nd and the 107th day parathormone was discontinued. On the 108th and 109th days 3 units were given daily; then 8 units from the 110th to the 158th day, and 20 units daily to the 168th day, when the dog was killed to terminate the experiment.

Blood calcium studies showed strikingly the progressive fall that occurred as the animal was decalcified. The serum calcium was 13.0 mg. per 100 cc. on the 12th day, 11.4 on the 28th, 10.9 on the 34th, 9.6 on the 42nd, and 8.1 on the 49th day. The dog at this time was rigid, dull, and had respiratory difficulties. On the 53rd day it had tremors and tetany, the serum calcium being 7.8 mg. per 100 cc. The tetanic symptoms may be taken as an indication of extreme exhaustion of calcium from the bones and soft tissues. At this time the forelegs were bowed outward, and the animal was weak and had difficulties in walking. It was decided to discontinue the parathormone and administer 650 mg. of calcium daily in order to preserve the animal's life. Between the 53rd and 60th day, when the dog was receiving the large calcium supplement, its clinical condition improved rapidly, the serum calcium rising to 9.0 mg. On the 60th day the calcium was discontinued and the serum calcium began to show a consistent drop reaching 7.8 mg. per 100 cc. by the 97th day. On the 108th day parathormone was again started at 8 units daily. The serum calcium remained fairly constant at about 8.0 mg. per 100 cc. or less. On the 168th day, when the experiment was terminated, and after the dog had been on 20 units for 10 days, the serum calcium was 7.8 and the serum phosphorus 6.4 mg. per 100 cc., although at first the animal had responded to the increased dose by a serum calcium as high as 10.4 mg.

The clinical condition of the dog makes an interesting study. On the 40th day its abdomen was distended, apparently as a result of hyperparathyroid hypotonia. Between the 48th and 53rd days there was difficulty in breathing, dullness and rigidity, with frequent tetanic seizures with spasmodic defecation and urina-

tion when the dog was handled. On the 53rd day respiratory failure developed during a tetanic seizure. At this time a serum phosphorus value of 10.7 mg. per 100 cc. was found. Artificial respiration restored the dog in several minutes, after which the muscles were relaxed. High phosphorus values were observed frequently, particularly after tetanic seizures, which varied in severity during the period that followed, which lasted for about 8 weeks, while the dog was not receiving any parathormone. There was rapid improvement after calcium therapy was initiated. The appetite improved, and the animal walked about the cage. The calcium was discontinued after about a week, but parathormone therapy was not resumed for 7 weeks longer, until the 107th day. The tremors reappeared together with spasmodic defecation, grunting respiration, and atony of the abdominal wall. Greater deformities of the forelegs developed, the plantigrade stance becoming worse. Shortly before the dog was killed, bloody stools and bloody diarrhea appeared, probably due to parathormone overdosage (without hypercalcemia).

Weight at autopsy was 2.9 kg. The dog had attained its maximum weight of 3.58 kg. 3 weeks previously. The animal had been greatly stunted, both humeri were rotated on their long axes, as observed in osteomalacia, and even on gross examination marked softening of the bones was evident. The internal organs showed nothing unusual in the gross.

On histological examination the ribs showed very extensive replacement of the marrow by cellular connective tissue. Embedded in it were a few islands of lymphoid marrow. In these islands the blood forming cells were for the most part well preserved, and red and white blood cells were maturing. The megakaryocytes were few and degenerated. The spindle connective tissue replacing the marrow was in places oedematous, and it contained a sprinkling of lymphocytes, polymorphonuclear leucocytes, some macrophages and plasma cells. A few lymphoid marrow cells could still be observed scattered through the connective tissue, which was quite vascular. Some osteoclasts were also observed in the marrow connective tissue. Very little remained of the original cortical bone, which had been resorbed, as evidenced by the enlarged vessel canals filled with fibrous tissue. One of the ribs examined showed a fracture through the cortex, and there was an attempt at repair by the formation of subperiosteal and endosteal callus. Throughout the fibrosed marrow a large number of osteoid and lamellar-bone trabeculae were observed. Many of these osteoid trabeculae were covered by osteoclasts, while the lamellar bone trabeculae were generally naked, and showed osteoclasts in considerable numbers in or near Howship's lacunae. At the costochondral junctions there was considerable osteoid-bone, and the intertrabecular connective tissue contained numerous osteoclasts. Many small cyst-like spaces were observed throughout the scarred marrow, but were most numerous at the costochondral junctions. They were lined by very flat endothelioid cells, and many were surrounded by lymphoid marrow. Some of these cysts were definitely blood-vascular spaces, containing red blood cells. These spaces were apparently related to stasis of marrow circulation. Some were possibly of

lymphatic origin. However, it seems to be impossible to trace definitely the origin of all of the cyst-like spaces.

Examination of longitudinal sections of the upper end of the humerus showed that the articular cartilage was normal. A very striking finding was the preservation of the marrow of the epiphysis, which was cellular and showed no fibrosis. The bone trabeculae of the epiphyses were thin, but not transformed. The epiphyses were therefore entirely free of *ostitis fibrosa*. The metaphyses and diaphyses, however, showed cortical resorption, osteoid-bone formation, numerous osteoclasts in Howship's lacunae, fibrosis and marked congestion of the marrow and enlargement of the vessels with marrow hemorrhage. There had been a pathological fracture of the humerus. The femur showed similar changes, and even to a greater degree. They were confined to the metaphysis and diaphysis. The femur also had a pathological fracture. The sections of several other tubular bones showed similar changes, but to a lesser degree.

Cross-sections from the diaphyses of the long tubular bones showed extremely enlarged vessel canals containing fibrous marrow. Some Howship's lacunae and osteoclasts were also seen. The haversian canals were lined by osteoblasts. Ground disks from the same regions also showed marked resorption.

The kidneys on the whole were well preserved, showing in places nests of lymphoid cells, a few sclerosed glomeruli, and calcium in some tubules. Numerous liver cells were replaced by fat. The other tissues showed nothing unusual. The specimens were not fixed or stained specially for calcium.

Summary.—This dog showed extensive resorption and fibrous replacement of the bone and marrow with considerable new bone formation (osteoid tissue). The fibrosed marrow was congested and contained cyst-like spaces. Deformities of the legs developed and histologically there was evidence of numerous pathological fractures. In spite of many evidences of severe hyperparathyroidism, the serum calcium showed consistent decline, apparently accompanied by lowered tissue calcium, leading eventually to tetany. The development of all of these consequences of hyperparathyroidism was no doubt enhanced by the calcium-poor diet, but cannot be attributed, of course, to the diet as such.

Dog 9.—Initial weight, 2.25 kg. The dog gained weight slowly but continuously to the termination of the experiment, 167 days later, when it weighted 5.35 kg. Calcium supplement, 200 mg. daily for the first 23 days (bone meal and calcium lactate), and 1300 mg. from the 24th to 27th day (calcium lactate). From the 28th to the 88th day the dog received daily 650 mg. of calcium (50 cc. of a 10 per cent solution of calcium lactate by stomach tube). Between the 89th and the 143rd day no supplementary calcium was given, then 100 mg. of calcium (bone meal and calcium lactate) were added and continued to the end of the experiment.

Four units of parathormone were injected during the first 6 days, and 8 units from the 7th to the 10th day. The serum calcium was 16.4 mg. per 100 cc. on the 10th day. Parathormone was omitted from the 11th to the 16th day, the serum calcium falling to 11.3 mg. on the latter day. From the 17th to the 20th day 6 units were given daily and the serum calcium rose to 12.4 mg. On the 21st and 22nd days the dog received no parathormone; on the 23rd and 24th, 8 units daily; none on the 25th and 26th. The serum calcium was 11.4 mg. per 100 cc. on the 24th and 11.0 on the 27th. From the 27th to the 43rd day the dog received 8 units daily; the serum calcium rising to 15.0 mg. on the 35th day and 15.5 on the 36th. The treatment being continued, serum calcium fell to 10.9 mg. on the 42nd day. On the 44th day the parathormone was increased to 12 units daily. The serum calcium was 12.1 mg. on the 45th day. On the 51st day, after some loss of appetite, the parathormone was decreased to 6 units daily, raised to 8 units daily on the 59th day, and continued at this dose to the 138th day. The serum calcium remained constant at normal values while the animal received 650 mg. of calcium daily (to the 88th day). During the period when no calcium supplement was given (88th day to 143rd day) the serum calcium dropped consistently. It was 10.0 mg. per 100 cc. on the 100th, 9.6 on the 103rd, 9.6 on the 112th, 8.5 on the 133rd, and 8.8 on the 140th day. On the 140th day the parathormone was raised to 20 units a day and continued at this dose until the termination of the experiment of the 167th day. The serum calcium rose to 9.9 mg. per 100 cc. on the 147th day, and was 10.1 on the 150th, 10.8 on the 154th day, 10.7 on the 161st day, and 10.6 on the 167th day.

Clinically this animal showed hypercalcemia and hyperparathyroidism on several occasions. The appetite was generally good and there was a progressive gain in weight. There was marked stunting of growth, the forelegs were bowed outwards, the bones were thin. The dog was well nourished at autopsy and otherwise showed nothing unusual in the gross.

On histological examination, the ribs showed extensive fibrous replacement of the marrow, but considerable lymphoid marrow still remained. The fibrous tissue of the marrow was oedematous. Marked resorption of the existing bone was evident, and numerous osteoclasts were observed in Howship's lacunae in the marrow connective tissue. Numerous trabeculae of osteoid-bone were observed near the costochondral junction. Some cyst-like spaces were present. The changes were of the same order, but somewhat less pronounced than in Dog 8. The long tubular bones also showed resorption of the compacta, enlargement of the vessel canals, which were filled with fibrous tissue. There was subepiphyseal cartilage osteoid-bone formation, and some fibrosis of the marrow. The epiphyses were free of these changes. Stained cross-sections and ground disks made of bone taken from the middle of the diaphyses of tubular bones showed enlarged vessel canals, and subperiosteal and subendosteal resorption. In the stained sections numerous osteoclasts were seen in the enlarged vessel canals, as well as some fibrous tissue and osteoid.

In a section of the lower jaw bone resorption, fibrosis, osteoclasts and cyst-like spaces were found, while a section from the calvarium showed similar changes and some osteoid-bone formation.

Nothing unusual was observed in the kidney, and no calcium deposits were found in sections stained by the v. Kossa method. None of the other soft tissues showed anything striking, and calcium deposits were found only in the stomach. The parathyroid glands seemed to have undergone simple involutional atrophy.

Summary.—This dog also showed the typical bone changes of chronic hyperparathyroidism. The histological picture satisfies the criteria established as necessary for the diagnosis of *ostitis fibrosa*. The absence of metastatic calcification in the tissues of this dog, in spite of the prolonged treatment, is significant. It seems to indicate that while metastatic calcifications are to be expected in acute hyperparathyroidism with hypercalcemia, the absorption of the calcium deposits is likewise to be expected in the stage of chronic hyperparathyroidism without hypercalcemia. (See also Dog 10.)

Dog 10—Initial weight, 1.05 kg. Weight at termination of the experiment, 185 days later, 4.8 kg. Greatest weight, 5.3 kg., was attained 19 days before the dog was killed. Calcium supplement, 560 mg. calcium daily for the first 5 days (bone meal and 200 mg. calcium daily from the 6th to the 105th day (bone meal and calcium lactate). Calcium was discontinued until the 162nd day, when 100 mg. of calcium daily was added and continued to the 185th day. The dog was injected with 2 units of parathormone daily for the first 7 days, 4 units daily to the 11th day, at which time the serum calcium was 15.2 mg. per 100 cc. From the 12th to the 24th day the dose was reduced to 2 units daily. The serum calcium on the 28th day was 12.7 mg. On the 30th day parathormone was increased to 8 units daily, but injections were discontinued from the 33rd to the 40th day because of loss of appetite. They were resumed on the 41st day at 6 units daily. The serum calcium was 14.7 mg. on the 42nd day, 12.5 on the 45th, 12.3 on the 53rd, and 11.5 mg. on the 60th day. On the 61st day parathormone was increased to 12 units daily. The serum calcium rose to 12.2 mg. on the 63rd, and 13.0 mg. on the 67th day, with associated loss of appetite. After omitting one dose of parathormone, it was decreased on the 69th day to 6 units daily until the 78th day, at which time the serum calcium was 11.6 mg. On the 79th day the injection of 8 units of parathormone was begun and this dose was continued to the 157th day. Serum calcium was 11.5 mg. on the 81st day, 10.7 on the 88th, and 11.6 on the 96th day. On the 106th day the supplementary calcium was discontinued, the parathormone dose being maintained at 8 units daily. The serum calcium was 10.8 mg. on the 118th day, 12.5 on the 112th, 10.7 on the 130th, 11.1 on the 151st, and 10.8 on the 158th day. On the 160th day the dose of parathormone

was increased to 20 units daily, but the serum calcium remained unchanged at 11 mg. on the 165th day, 11.2 on the 168th, 11.0 on the 172nd, 11.2 on the 179th, and 11.2 on the 185th day, when the dog was killed to terminate the experiment.

On autopsy the dog was well nourished. Otherwise the autopsy showed nothing unusual in the gross, except that there was slight bowing of the bones. The dog was markedly stunted. The changes in the bones of this dog were essentially the same as in No. 9. Histological examination of the soft tissues revealed nothing unusual. The splenic pulp was somewhat fibrosed and some of the liver cells were fatty. Tissues appropriately fixed and stained showed a striking absence of calcium. This was in significant contrast with our observations of metastatic calcifications in dogs dying of acute hyperparathyroidism. Only a few kidney tubules showed some calcium in the lumina.

Summary.—The tissue changes and their significance were the same as in Dog 9. Bone lesions characteristic of *ostitis fibrosa* were produced as a result of prolonged treatment with parathormone.

DISCUSSION

We have produced *ostitis fibrosa* in young growing puppies. We believe that the period of active skeletal growth is more suitable for the development of this condition. During the period of growth the demand for calcium is great, but even on a liberal calcium intake parathormone interferes with calcium utilization, and with the formation of normal bone, the calcium balance being negative on appropriate dosage. An analogy is found in the well-known requirement in the standardized procedure for the production of experimental rickets, the development of which is favored by rapid growth. The changes that we have observed in experimental hyperparathyroidism are not of the type that are caused by low calcium diets. On such diets animals are not stunted, provided the diets are not deficient in vitamins; no marrow destruction and fibrosis are found. These lesions seem to be specific effects of parathormone treatment. The decalcification in hyperparathyroidism is not only more rapid than in simple calcium deprivation, but perhaps because of the greater rate of decalcification the secondary effects develop which serve to distinguish the two conditions.

That parathormone affects the lymphoid marrow cells directly, in addition to its effects on the bone, was observed in all dogs dying of acute hyperparathyroidism. We believe that during acute hyper-

parathyroidism destructive changes appear also in other lymphoid tissues. There seems to be no doubt that the severe marrow fibrosis is in part an expression of the healing of the injured marrow during prolonged parathormone therapy.

It may well be that the immediate antecedent of experimental ostitis fibrosa is a condition, perhaps related to the disturbed acid-base equilibrium, which may also be caused by other agents than parathormone. However, it seems essential that the action be continuous, and that the condition which it causes be maintained for long periods without endangering the life of the experimental animal, or a certain minimum of well-being. Parathormone is specific in the sense that it satisfies these requirements.

In acute experimental hyperparathyroidism, metastatic calcifications are generally observed, associated with hypercalcemia. We have confirmed this observation. In some of our animals, the stage of acute hyperparathyroidism was succeeded by chronic hyperparathyroidism, without hypercalcemia. In these animals, when they came to autopsy metastatic calcifications were absent or nearly absent. We suggest that metastatic calcifications had been present in these animals earlier, and were resorbed during the chronic stage of hyperparathyroidism.

It has been widely realized that parathormone effects are to be judged by calcium excretion, particularly in the urine, rather than by a rise of serum calcium. And yet, absence of parathormone effects has been frequently inferred from the absence of hypercalcemia. It therefore remains necessary to emphasize that parathormone effects on bone may be observed, as our protocols prove, even when the calcium values are normal or lower than normal.

The lesions produced in these young animals were of the order observed in v. Recklinghausen's disease (ostitis fibrosa cystica, or the hypostotic-porotic form of osteodystrophia fibrosa). It is well known that clinically most of the cases of ostitis fibrosa cystica of v. Recklinghausen come to notice before middle age, the condition probably having developed for many years without clinically prominent symptoms. Hirsch, basing his conclusions on X-ray experience, believes that most of these cases begin early in life (50).

One of the very striking features observed in our young dogs whose

epiphyses were as yet not fused with the diaphyses, and in whom epiphyseal cartilage plates were still present, was the absence of fibrous osteodystrophy in the epiphyses. The epiphyses at most showed some simple bone atrophy, but marrow fibrosis and active bone resorption, with Howship's lacunae and osteoclasts, were absent. The lesions were most prominent in the metaphyses, where active new bone formation would normally take place. It seems quite definite that bone injury and marrow fibrosis observed by us in these experiments are not dependent upon vascular changes, as the epiphyses are also exceedingly vascular during the growing stage and would share in the changes if the vascular factor were decisive.

The presence of osteoid tissue as a requisite for establishing the diagnosis of *ostitis fibrosa* seems to have been given too much significance. In advanced clinical cases with considerable deformity, it appears almost always. Its appearance, we believe, is related to the physiological need of reinforcing bones subject to decalcification. In our dogs we observed osteoid tissue formation when decalcification was most severe, and when there was definite need for repair, as for instance in response to a gross fracture. We conceive that rapid decalcification, when gross fractures do not occur, leads to a weakening of the bone at innumerable points, especially if the animal is active. In view of the stresses and strains exerted at these points, microscopical fractures may be conceived, in the repair of which osteoid is produced. Osteoid may also be formed on greatly-thinned bone as an expression of the need to repair.

In regard to the presence of cysts in our dogs with advanced fibrous lesions, these are, we believe, and as many pathologists have observed in clinical material, due for the most part to circulatory stasis that results from the distortion and scarring of the marrow incident to the development of the *ostitis fibrosa*.

In dogs dying of acute hyperparathyroidism we observed reduction in the size of the external parathyroids to about one-half their usual size. Microscopically, the reduction in size was found related to a diminution in the size of the parenchymatous cells and to their distortion. We interpret this picture as evidence of functional involutional atrophy due to substitution therapy.

SUMMARY

These experiments have shown that parathyroid extract (parathormone Collip) can be injected into puppies in increasing amounts for long periods without fatal results. Thus time is allowed for bone changes to develop. Long continued injection leads to progressive decalcification and resorption of the existing bone, to fibrous replacement of the marrow, and to the production of the other features characteristic of *ostitis fibrosa*. Deformities eventually appear. It is safe to assume that the bone changes produced by hyperparathyroidization have the same pathogenesis as those observed in clinical cases believed to be instances of hyperparathyroidism—that is, cases with a negative mineral balance and decalcification of the skeleton.

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EXPLANATION OF PLATES

PLATE 35

FIG. 1. Rib of Dog 9 showing costochondral junction. The marrow is fibrosed, some islands of lymphoid marrow are still present. A few cysts are observed. Magnification 18X. Stained with hematoxylin and eosin.

FIG. 2. Rib of Dog 10. The marrow is fibrosed and contains numerous osteoclasts. Cysts are present. Magnification 20X. Stained with hematoxylin and eosin.

FIG. 3. Rib of Dog 8. The marrow is fibrosed and numerous cysts are seen. Considerable newly formed trabeculae are present. In places there is disappear-

ance of the cortical bone. Magnification 10 \times . Stained with hematoxylin and eosin.

FIG. 4. Rib of Dog 8 showing remaining islands of lymphoid marrow, many of which surround cysts. Fibrosis of the marrow, thinning and transformation of the cortex are more evident than in Fig. 3. Magnification 20 \times . Stained with hematoxylin and eosin.

FIG. 5. A higher power picture from same rib showing marrow fibrosis, cysts, and newly formed fiber-bone. Magnification 150 \times . Stained with hematoxylin and eosin.

FIG. 6. Shows part of the epiphysis (above) and metaphysis (below) the epiphyseal cartilage plate of femur of Dog 8. There is no endochondral-bone formation at the epiphyseal cartilage plate. Lymphoid marrow is observed in the epiphysis, where marrow fibrosis is absent. In the metaphysis marrow fibrosis and numerous cysts are seen. Magnification 16 \times . Stained with hematoxylin and eosin.

PLATE 36

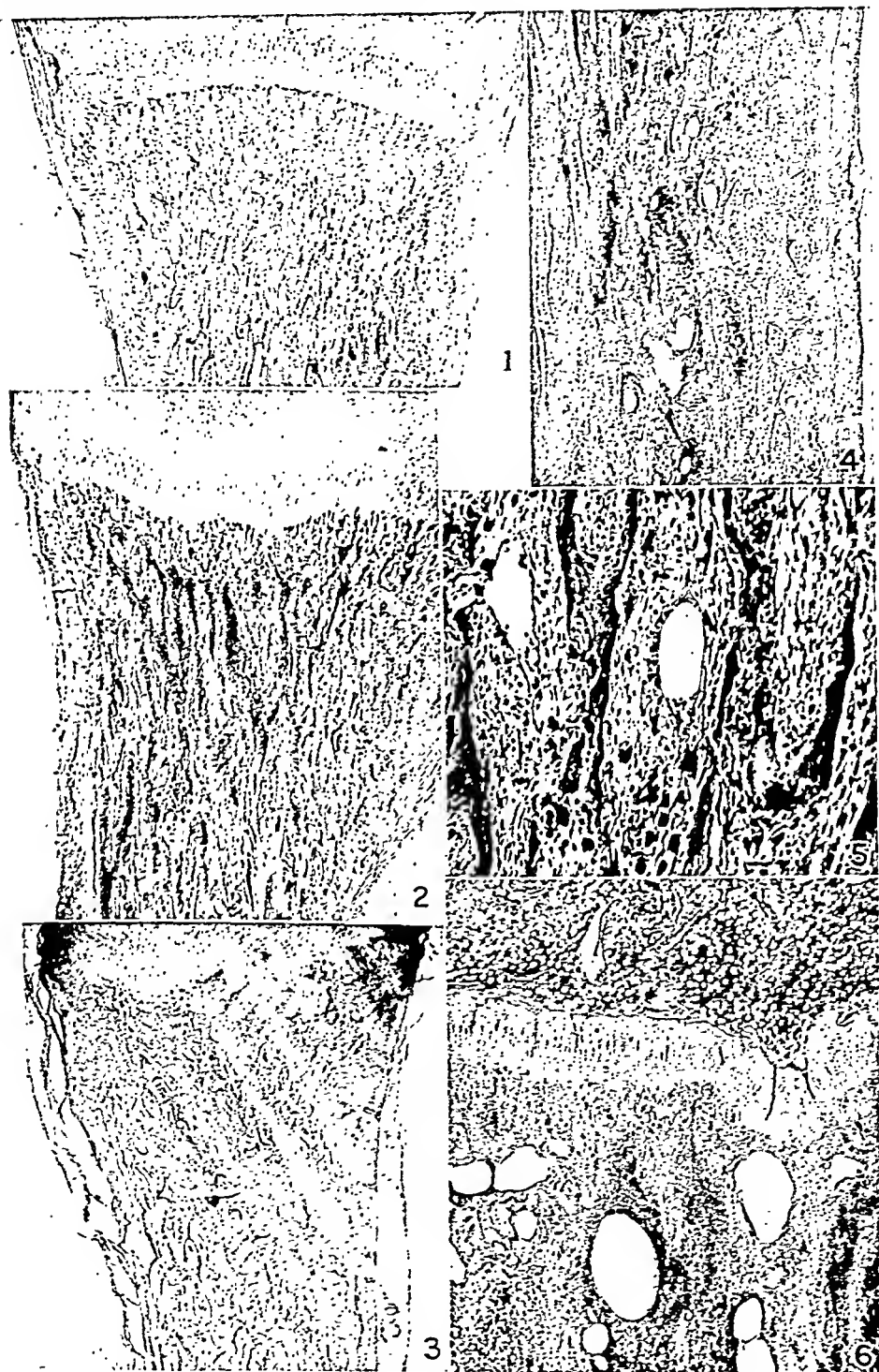
FIG. 7. The diaphysis of the femur of Dog 8 showing considerable marrow fibrosis with resorption of bone. Magnification 20 \times . Stained with hematoxylin and eosin.

FIG. 8. Osteoid tissue in the fibrosed marrow of the femur of Dog 10. Magnification 60 \times . Stained with hematoxylin and eosin.

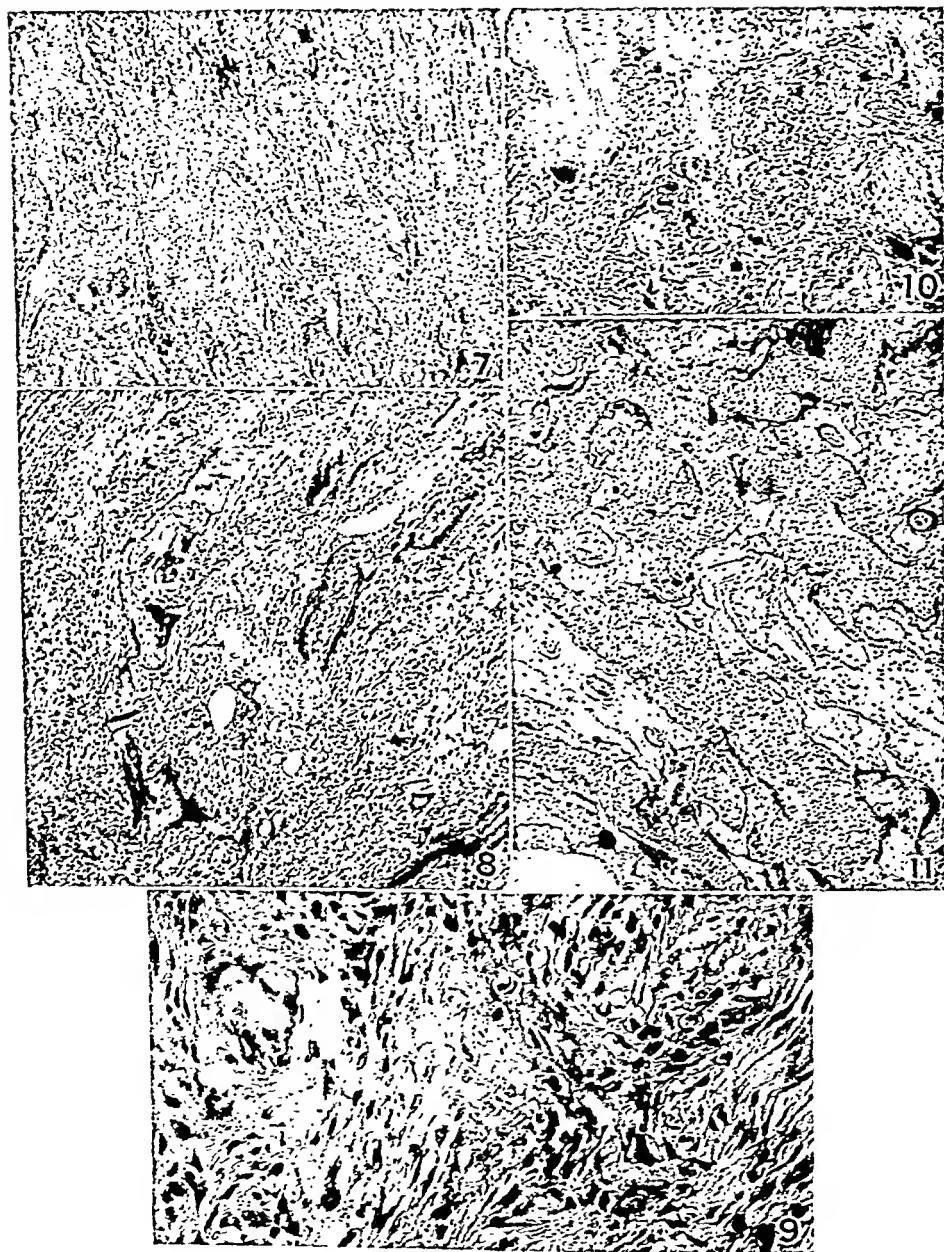
FIG. 9. Fiber-bone formation in the fibrosed marrow. Magnification 300 \times . Stained with hematoxylin and eosin.

FIG. 10. Shows extensive osteoclastic resorption below the epiphyseal cartilage plate of the humerus of Dog 9. Magnification 85 \times . Stained with hematoxylin and eosin.

FIG. 11. Shows resorption and fibrosis of bone from calvarium of Dog 10. Magnification 85 \times . Stained with hematoxylin and eosin.



(Jaffe and Bodansky: Experimental fibrous osteodystrophy)



(Jaffe and Bodansky: Experimental fibrous osteodystrophy)



A COMPARISON OF THE CHEMICAL ALTERATIONS IN THE BLOOD OF RATS INFECTED WITH PATHOGENIC AND NON-PATHOGENIC TRYPANOSOMES

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In a recent study (1) of some of the factors involved in acute infections with *Trypanosoma equiperdum* in rats it was found that the CO₂ capacity of the serum was lowered, the lipid phosphorus, and lecithin, increased, and the liver glycogen greatly diminished or absent. The blood sugar, on the other hand, remained at a normal level until late in the disease. Cholesterol remained unchanged in amount, while the non-protein nitrogen and uric acid increased only subterminally with the partial breakdown of the kidneys.

It seemed of interest to investigate the factors which had proved to be influenced in the pathogenic infection, in infections due to the non-pathogenic *Trypanosoma lewisi*, to determine a possible injurious effect of this parasite. The blood sugar in rats infected with this organism has already been reported upon *in extenso* (2), and found to remain normal.

The methods are those that were used in the previous work. The results for the lipid phosphorus, the calculated lecithin, and the liver glycogen, are given in Table I. Table II summarizes the experiments for the determination of the CO₂ capacity, and Table III contains a comparison between the fatal and the non-fatal infections, in respect to these three factors.

The data for the 21 animals in Table I are arranged to illustrate the rise and fall in number of the trypanosomes throughout the infection. The determinations were made from animals with increasing numbers of trypanosomes up to 450,000 per cubic millimeter (No. 11): the remaining animals were those in which the crisis was passed. The lipid phosphorus was determined, and the lecithin calculated,

in normal animals before the infection was given, and again when the infection had reached the stage indicated by the trypanosome counts. The percentage of variation between the two determinations is also given.

This variation is positive in 9 cases and negative in 4. The normal range for lecithin, based on the results of this and the previous paper, seems to lie in the rat between about 170 and 250 mg. per 100 cc. of blood. In 4 of the animals only a single determination was made, and all of these lie within the normal range (Nos. 5, 12, 14, and 19). In one animal (Rat 6) the upper limit is transcended, and the variations throughout, both positive and negative, are relatively slight and without the marked upward trend which is apparent in the infections with *T. equiperdum*. These figures for rats are slightly higher than those for human blood as determined with this method by Rosen and Krasnow (3); they found the range to be from 160 to 240 mg.

The figures showing the amount of liver glycogen, calculated as glucose per gram of liver, are also included in Table I. This arrangement does not imply any connection between the two factors. Glycogen is found to be present in significant amounts in every case, and does not vary with the rise and fall of the parasites. This finding is in contrast with the results of Regendanz and Tropp (4). These workers have published figures for 4 rats infected with *T. lewisi*, which show, they believe, that the glycogen is significantly lowered. Their animals had been splenectomized in order to increase the number of organisms in the blood. It is impossible to tell, however, to what extent such an increase has actually occurred, since they do not publish any trypanosome counts; nor are their figures for the glycogen content much lower than "normal," especially in view of the great variations to which this constituent is subject.

Table II presents the CO₂ capacities of the serum of 12 infected rats. All of these animals were taken for study at the time when the parasites were in the stage of rapid increase. The values found are all within the normal range. They show that the rats do not suffer from an acidosis during the infection, although acidosis is present from an early stage in infections with *T. equiperdum*.

TABLE I

Lipoid Phosphorus, Lecithin, and Liver Glycogen, as Glucose, in Rats Infected with Trypanosoma lewisi

Rat No.	Before infection		After infection		Percentage variation	No. trs. per cu.mm.	Glucose. Mg. per gram of liver
	Lipoid p. Mg. per 100 cc. blood	Lecithin. Mg. per 100 cc. blood	Lipoid p. Mg. per 100 cc. blood	Lecithin. Mg. per 100 cc. blood			
1						50,000	12.3
2						75,000	12.0
3	8.9	222.5	10.1	252.5	+13	92,500	
4	8.1	202.5	8.5	212.5	+5	125,000	17.6
5			8.65	216.2		140,000	13.3
6	9.85	246.2	11.6	290.0	+18	150,000	8.1
7	8.25	206.2	10.0	250.0	+21	167,000	8.0
8						200,000	18.0
9	8.6	215.0	9.9	247.5	+15	317,500	7.1
10	9.05	226.2	9.9	247.5	+9	342,000	4.8
11	9.1	227.5	9.0	225.0	-1	450,000	9.8
12			9.5	237.5		200,000	11.0
13	8.9	222.5	8.65	216.2	-3	130,000	35.0
14			8.3	207.5		125,000	9.5
15	8.6	215.0	8.3	207.5	-3	105,000	6.6
16	9.2	230.0	9.3	232.5	+1	100,000	20.4
17	9.15	228.7	8.6	215.0	-6	100,000	
18						100,000	15.5
19			9.05	226.2		62,500	6.1
20	8.8	220.0	9.8	245.0	+11	62,500	
21	7.35	183.7	7.75	193.7	+5	25,000	

TABLE II

Carbon Dioxide Capacity of Serum in Rats Infected with Trypanosoma lewisi

Rat No.	Trypanosomes per cu.mm.	CO ₂ capacity. Vol. per cent
1	100,000	56.0
2	137,000	56.0
3	150,000	64.6
4	175,000	71.1
5	180,000	60.7
6	200,000	63.6
7	200,000	69.1
8	225,000	68.3
9	230,000	69.1
10	237,500	62.6
11	250,000	58.8
12	275,000	64.4

DISCUSSION

Table III gives a comparison of the pathogenic and non-pathogenic infections. In *T. lewisi* rats the lecithin varies from the normal -6 per cent to +21 per cent, and shows an average increase of +6.5 per

TABLE III

Percentage Variations of Lecithin, and Average Liver Glycogen Content, as Glucose, throughout Infections with Trypanosoma lewisi and Trypanosoma equiperdum

	Lecithin	Glucose. Mg. per gram of liver
<i>T. lewisi</i>	-6 per cent to +21 per cent avg. + 6.5 per cent (9 increases, 4 decreases)	12.6 mg. (no negative cases)
<i>T. equiperdum</i>	+8 per cent to +99 per cent avg. 35.6 per cent (11 increases, no decreases)	1.8 mg. (7 negative cases out of 11)

Variation in CO₂ Combining Capacity at Various Concentrations of Trypanosoma lewisi and Trypanosoma equiperdum per Cubic Millimeter of Blood

	No. trypanosomes	CO ₂ combining cap. Vol. per cent
<i>T. lewisi</i>	137,500	56.0
<i>T. equiperdum</i>	137,500	37.0
<i>T. lewisi</i>	200,000	69.1
<i>T. equiperdum</i>	200,000	13.6
<i>T. lewisi</i>	237,500	62.6
<i>T. equiperdum</i>	225,000	45.0
<i>T. lewisi</i>	275,000	64.4
<i>T. equiperdum</i>	275,000	35.0

cent; 9 of the animals showed increases and 4, decreases. With *T. equiperdum* all the animals showed increases, ranging from +8 per cent to +99 per cent, and averaging +35.6 per cent. The same difference is shown in the comparison of the glycogen. With *T. lewisi* this constituent was present to an average extent of 12.6 mg. of glucose per gram of liver, and no animals were found in which it could

not be demonstrated; in the livers of *T. equiperdum* animals, on the other hand, it was absent in 7 out of the 11 cases, and averaged for the series 1.8 mg. per gram of liver. The CO₂ capacities are contrasted in 4 sets of animals in which the concentrations of trypanosomes per cubic millimeter were similar. In each case the *T. lewisi* animals show a normal, and the *T. equiperdum* animals an abnormal, capacity.

In general, the table, together with the others in this and the previous paper, shows that the action of *T. equiperdum* on these three factors is not due to the influence of its numbers, since equal numbers of *T. lewisi* are without effect, but to some action which it has upon the host. To apply the term "toxin" to this effect is simply a begging of the question, since no completely acceptable evidence for the existence of such a substance has ever been adduced.

The possibility must be kept in mind that counting the trypanosomes in the peripheral blood may not be a valid method of comparing the extent of the two infections, since large numbers of the pathogenic organisms might be retained in, and have their effect upon, the inner organs. Work now in progress shows that such an "inner action" does occur when guinea-pigs are infected with *T. equiperdum*, in that a severe anemia appears in these animals relatively long before the parasites are demonstrable in the blood stream, or while their concentration is extremely low.

By the study of the factors here discussed it should be possible to determine whether "pathogenic" strains of *T. lewisi* exist. At present, the evidence for the pathogenicity of this organism is not convincing; especially is this so since Marmorston-Gottesman and Perla (5) have shown that death of normal animals infected with *T. lewisi* may really be due to a concomitant infection with *Bartonella muris*.

SUMMARY

Blood samples from rats infected with *Trypanosoma lewisi* give normal values for lipid phosphorus, lecithin, CO₂ combining capacity, and liver glycogen.

When these results are compared with the results of similar experiments with *T. equiperdum* infections, on the basis of the concentration

of trypanosomes in the blood, it is found that the pathogenicity of the latter organism does not depend upon its numbers as affecting the blood, but that it must be in some other way injurious to the host. Whether the injury is due to a true toxin, an endotoxin, or mechanical interference is not yet known.

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STUDIES IN THE COMMON COLD

IV. EXPERIMENTAL TRANSMISSION OF THE COMMON COLD TO ANTHROPOID APES AND HUMAN BEINGS BY MEANS OF A FILTRABLE AGENT

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In this study of the upper respiratory infections usually grouped under the heading of common cold, our primary interest has been in etiology. In earlier work (1, 2), two groups of organisms were investigated for possible causative relationship: (a) the bacteria more readily cultivated from the upper respiratory tract and (b) the group of Gram-negative, filter-passing anaerobes first described by Olitsky and Gates (3) and Olitsky and McCartney (4) and more recently in studies by Branham (5), Noble and Brainard (6) and others.

In our work a number of normal individuals were taken and by serial cultures, over periods of several months, the basic bacterial flora of their noses and throats was determined. Concurrently detailed observations were made of quantitative and qualitative changes occurring in this flora in the course of colds. The bacteria included in the first study (1) were streptococcus, hemolytic and non-hemolytic; *B. Pfeifferi*, including the hemolytic forms; Gram-negative cocci; diphtheroids; *Staphylococcus albus*, *aureus* and *citreus*; pneumococcus and other occasionally encountered miscellaneous organisms. In the second study (2) the various types of filter-passing anaerobes referred to above were included.

From the results obtained in this work we were led to conclude that none of the organisms in the first group are of primary etiological significance, although some of them are of importance as secondary invaders at times and that the filter-passing anaerobes constitute

part of the normal flora of the upper respiratory tract and do not seem to bear a causative relationship to colds.

Having failed in these attempts to assign a causative rôle to bacteria in these two groups, we were led logically to testing the hypothesis that colds may be initiated by a filtrable virus. The present report deals with an investigation of this possibility, using the medium of ape and of human transmission experiments.

In 1914 Kruse (7), later Foster (8) and more recently Olitsky and McCartney (4) reported successful transmission experiments using filtrates obtained from the nasal secretions of individuals suffering from colds. However, the following workers, Schmidt (9), Williams and her co-workers (10), Branham and Hall (11) and Robertson and Groves (12) failed in their efforts to confirm these results. In general the method used by these workers has been the intranasal inoculation of human volunteers.

In view of the difficulty of securing appropriate human subjects and particularly of effectively quarantining them, it was felt that animals would be far more satisfactory for transmission experiments. In searching about for such suitable animals it was learned that anthropoid apes were supposed to catch colds. Accordingly, curators of zoological gardens, and dealers and workers with these animals were sought out for confirmation of this report. It was found very soon that all workers with the higher apes were unanimously of the opinion that these animals readily caught colds from human beings similarly afflicted, and further that these colds were more or less similar clinically to those observed in man.

I. Suitability of Chimpanzees for Experimental Work in Upper Respiratory Infections

Having satisfied ourselves with respect to the probable suitability of the anthropoids for our purposes, we began acquiring chimpanzees. Young animals, aged 2 to 4 years and weighing from 15 to 30 pounds were chosen. In general, at these ages, the animals are quite gentle and with kindly training very rapidly become most cooperative. In the course of the first year's work we were able to collect suitable chimpanzees to the number of six. At the end of the first year the

colony numbered eight and during the last year two more animals have been added.

The animals have been kept in cages in rooms where a constant temperature of approximately 80°F. has been maintained by thermostatic control. They have been protected very carefully from contact with outsiders and all workers have been required to wear masks upon entering. When the animals have been used for transmission experiments they have been transferred from the stock room to special quarantine rooms where extreme precautions have been taken to exclude all possible infection from without. In general, everything entering the rooms including most of the food is sterilized. All workers wear sterile gowns, masks, caps and rubber gloves. That this system is quite effective has been shown by the fact that no accidental infection has ever occurred among the quarantined animals although there have been many such spontaneous infections among the animals in the stock room.

General Observations on Susceptibility of Chimpanzees to Colds.—Very early in our work we learned to our sorrow the truth of the reports regarding the susceptibility of the anthropoids to human upper respiratory infection. While our animal quarters were still in process of construction one of the first two chimpanzees acquired by us, while boarding at the animal dealer's, caught a cold from his keeper. The infection went over to what appeared to be a pneumonia and the animal finally succumbed. Post mortem examination showed bronchopneumonia and from the lungs there were recovered hemolytic streptococci, Pfeiffer's bacillus, *B. alkaligenes* and a Gram-negative anaerobic filter passer.

Since that time we have had abundant opportunity to observe the readiness with which these animals catch colds from human beings afflicted with these infections. When the men handling the animals have had colds the apes in the stock room frequently have contracted the infection even though the men have always worn masks. Colds of this type also spread rapidly from ape to ape. Such spontaneous infections have never occurred in the quarantine rooms as noted above, as in addition to the precautions already mentioned rigorous exclusion of workers with colds has been maintained.

When a chimpanzee catches a cold, the clinical picture presented is very similar to that observed in a human child.

At first there is a small amount of glairy mucus in the nostrils. By the end of the first day the animal usually appears quite sick, lassitude being fairly striking. The eyes are puffy and drooping, there is a moderate to profuse nasal discharge of thin mucus which runs down over the upper lip; there is definite nasal obstruction which makes it difficult for the animal to take liquid food and the breathing becomes audible. There is occasional sneezing and cough. The appetite is usually moderately impaired, rarely there has been diarrhea. Usually there is no elevation of temperature. By the second or third day the nasal discharge becomes mucopurulent. The throat at times has appeared inflamed. By the fourth or fifth day the animal is usually much better and the discharge and nasal obstruction become much less. Recovery is usually complete in a week to 10 days. Occasionally there is a persistence of cough for several days resembling the bronchitis so frequently noted as a complication of colds. Occasionally, also, there has been a persistence of purulent nasal discharge, for days or weeks, suggesting a chronic paranasal sinusitis; this has been particularly true of one of the animals which has a large polypoid growth in the posterior naso-pharynx.

Of considerable interest to us have been the findings with respect to the animals' apparent immunity succeeding colds. Rarely have they been given or have they caught colds in less than 3 months after having a previous infection, and in most cases the interval between infections has been longer. This has suggested the possibility that there is a period of insusceptibility or of immunity of 3 or 4 months' duration, in these animals, succeeding colds. As a result of these findings we no longer attempt transmission experiments during the 3 or 4 months that follow a cold, as a negative result would be of little significance. One important exception to the foregoing has been noted. In December, 1928, three of our animals acquired acute upper respiratory infections 3 or 4 weeks after having had colds. Clinically, however, these infections closely resembled the influenzal type which was then epidemic and from which our animal men were suffering. Although very slight, this evidence points to the assumption that influenza and the common cold are separate entities.

The Bacterial Flora of the Upper Respiratory Tracts of Chimpanzees.—Soon after acquiring the apes a study was commenced to determine the bacterial content of their noses and throats. Swab cultures were taken from the nose and posterior naso-pharynx and were plated upon fresh rabbit's blood agar. The percentage incidence of the bacteria present in health is shown in Table I. For comparison we have

included in the table our earlier finds, 1924-25, in the case of man (1); the cultural methods used were the same in both cases (Table I).

It will be noted that the bacteria of the throat are surprisingly similar in man and ape. The usual basic organisms, non-hemolytic streptococci and Gram-negative cocci are identical in incidence. *B. pfeifferi* and hemolytic streptococci are higher in the ape than in man. *B. coli* appears in the ape flora as a result of the habits of the animal. Other differences are of slight degree only.

In the nasal cultures, staphylococci which are usually the predominating organisms in man are the same in incidence in the ape.

TABLE I

Comparison of Percentage Incidence of Bacteria in Noses and Throats of Normal Chimpanzees and Humans

		Organisms												
		Gram-negative cocci	Streptococcus non-hemolytic	Bacillus "X"	<i>B. pfeifferi</i>	Diphtheroids	Large Gram-positive cocci	<i>Staphylococcus albus</i>	Streptococcus hemolytic	<i>Staphylococcus aureus</i>	Pneumococcus	<i>B. coli</i>	Strepto-dacillus	Total cultures
Nose	Ape	17	60	0.6	13	21	8	95	1.2	26	1.2	4	15	169
	Man	1	7	0.0	0	79	0	92	0.4	36	0.0	0	0	265
Throat	Ape	99	99	21.0	92	11	5	31	45.0	9	0.6	13	0	159
	Man	99	99	49.0	47	45	45	40	17.0	14	2.0	0	0	265

Diphtheroids, very characteristic of the human nasal flora, are rather lower in percentage incidence in the ape. Strepto-bacilli seem to be peculiar to the ape and outside of the accidentally present *B. coli* seem to constitute the only qualitative bacterial difference. The higher percentage in the noses of apes of certain organisms such as Gram-negative cocci, non-hemolytic streptococci, *B. pfeifferi*, etc., which are usually regularly present in the throats of both man and ape are probably due to anatomical differences.

Gram-negative, filter-passing anaerobes also have been cultivated from nasal washings obtained from apes. Careful study of the incidence of these organisms has not been attempted because of the difficulty of securing washings.

Summarizing: (1) Chimpanzees are susceptible to colds when exposed to such infections in humans. (2) The clinical manifestations noted when the animals catch cold closely resemble those seen in man. (3) The bacterial flora of the nose and throat of these animals is very similar to that of man.

From the foregoing it seems clear that chimpanzees are suitable animals for the experimental transmission of colds.

II. Transmission Experiments with Chimpanzees

Having assured ourselves of the suitability of chimpanzees for our purposes we proceeded with attempts to transmit colds from human sufferers to apes by means of filtered nasal washings.

Methods.—Animals to be used were placed in the strict quarantine described above and were held there for several days before being used. This was done for the purpose of testing the efficiency of the isolation and for the exclusion of possible latent respiratory infection.

At the conclusion of this period of preliminary observation, which lasted 5 days or longer, individuals suffering from suitable colds were sought out. The type of cold selected was one of not more than 24 hours' duration and of moderate severity. A special effort was made to exclude colds that did not conform strictly to the classical clinical types, such, for example, as the so-called "grippy" varieties in which fever or other constitutional symptoms were manifest.

Nasal washings were obtained from the subjects with colds by gently running slightly warmed stock buffered broth (pH, 7.6) into their nostrils (5 to 10 cc. per side) and thence out through the mouth; 10 to 20 cc. of the broth was next gargled and added to the nasal washing. The material thus collected was shaken vigorously together with glass beads to break up the clumps of mucus. It was then passed through a Berkefeld V candle. The unfiltered material was cultured aerobically upon blood plates. The filtrate was cultured aerobically and anaerobically upon blood agar plates and blood broth and in the Smith-Noguchi medium to determine its sterility and the presence of filter-passing anaerobes. Further, as a control measure, 0.25 cc. of filtrate was injected intracerebrally or intracisternally in rabbits to exclude the presence of herpes virus.

As soon as possible after the filtration of the nasal washings, 1 cc. of the filtrate was injected with careful aseptic precautions into each nostril of the quarantined chimpanzees. For each experiment it was customary for us to collect two nasal washings at intervals of a few hours and to make two intranasal inoculations in each experimental animal with the filtrates obtained from these. The time elapsing between the collection of each washing and its injection into the ape was usually less than 1 hour.

Results.—In the chimpanzee experiments performed during the last 2 years we have had 44 per cent of successful transmissions by means of filtrates. A summary of these appears later. In positive experiments, the first symptoms of the cold have appeared within 36 to 48 hours following inoculation. A typical successful transmission experiment is shown in Fig. 1. On this chart appear both the clinical and bacteriological findings. The time of inoculation, the incubation

inoculation
↓

Ape	Date: October:	30	31	6	12	13	14	15	16	17	18	19	20	21	28
<u>Clinical</u> :-	Well	+	+	+	+	+									+
	Mucus in nose						+	+	+	+	+	+	+	+	
	Nasal Discharge						+	+	+	+	+	+	+	+	
	Nasal Obstruction						+	+	+	+	+	+	+	+	
	Sneezing														
<u>Cultures</u> :	Cough									+					
	Red throat														
	Appetite poor														
	Diarrhea														
Nasal-	Gram-neg. Cocci					+	#		#			+	+	#	○
	Non-hemol. Strep.			○	+	+	#								
	<i>B. Pfeifferi</i>								○				+	+	○
	Diphtheroids			#	#	○	○								#
	<i>Staph. Albus</i>	●		●	●	○	●	#	+			○	+	#	#
	<i>Staph. Aureus</i>														●
Naso-pharyngeal-	<i>Pneumococcus</i>						○	●	●	○		○	○	○	
	Gram-neg. Cocci	●	○	○	○	○	○	●	○	○	●				○
	Non-hemol. Strep.	○	○	○	○	○	○	○	○	○	○				
	<i>B. Pfeifferi</i>	#	#	#	#	○	+	#	#	#	#				#
	Hemol. Strep.	#	#	+				#							+
	<i>Pneumococcus</i>							○	#						●

FIG. 1. Chart showing development of symptoms in an experimental cold after intranasal inoculation of a chimpanzee with filtered nasal washings from a human cold. The bacterial flora in nose and throat before, during, and after is shown graphically. Solid circles indicate predominating organism, blank circle the next most numerous, plus signs indicate the remainder.

period and the sequence of symptoms are shown graphically in the upper portion and are self-explanatory.

On the lower half of the chart are recorded the bacterial findings and these are of considerable interest. It will be noted that coincident with the initiation of cold symptoms there is a definite alteration in the flora. This is most striking in the nose cultures, where pneumococci (Type IV) have become the predominating organism, and *B. Pfeifferi* has become conspicuous. In the throat cultures, pneumococci suddenly appear.

Alterations of this type, in which pneumococci have suddenly ap-

peared in the nose and throat cultures, or where *B. pfeifferi* and occasionally hemolytic streptococci have spread to the nose at the start of colds, have been present almost always in both the induced and the accidental infections in apes. In our studies of the flora in humans both in our previous, and the present work, we have not observed this phenomenon. The contrast between the slight qualitative changes in man and the striking alteration in the ape is shown in Table II.

TABLE II

Comparison of Percentage Incidence of Bacteria in Noses and Throats of Chimpanzees (a) during Normal Periods and (b) in the Course of Colds

		Organisms												
		Gram-negative cocci	Non-hemolytic streptococcus	Bacillus "X"	<i>B. Pfeifferi</i>	Diphtheroids	Large Gram-positive cocci	<i>Staphylococcus albus</i>	Hemolytic streptococcus	<i>Staphylococcus aureus</i>	Pneumococcus	<i>B. coli</i>	Strepto-bacillus	Number of cultures
Nose	Normal Colds	17	60	0.6	13	13	8	95	1.2	26	1.2	4	15	169
		38	33	0.5	59	9	2	87	5.0	11	49.0	10	25	172
Naso-pharynx	Normal Colds	99	99	21.0	92	11	5	31	45.0	9	0.6	13	0	159
		100	99	17.0	94	9	8	18	36.0	2	14.0	21	0	104

Early in the course of the work control experiments were begun, intranasal inoculations of plain broth and of heated filtrate being used. These were soon given up as it was believed that filtered nasal washings from normal individuals who were free from respiratory infection would be more suitable for control purposes. In order to reduce the likelihood of including carriers of the active agent, as sources of normal nasal washings, the summer months were selected for this group of experiments, colds being at a minimum in this season.

The animals were quarantined in the usual manner and after the customary preliminary period of observation they were inoculated with the filtered nasal washings, the same procedure which had been used for the transmission experiments being carefully followed throughout. As a source of the normal nasal washings healthy individuals who had had no colds or sequelae for at least 3 or

4 months and who had had no known recent exposure to current colds were used. Eight experiments were done in all.

Following these inoculations no change in the health of the animals was observed. There was an entire absence of even small amounts of nasal mucous discharge and no constitutional manifestations whatever were observed. In marked contrast with the characteristic alterations in the bacterial flora of the noses and throats of animals suffering from experimental or spontaneous colds, no changes were noted in these control animals.

The filtered nasal washings, obtained from individuals with colds and used to transmit these infections to apes, contained Gram-negative filter-passing anaerobes in a high proportion of cases. This is as it should be, if, as we believe, these organisms constitute part of the basic flora of the nose and throat. The fact that these organisms were present in 75 per cent of the control (normal) filtrates, which caused no symptoms whatever in the animals inoculated, provides further evidence that they have very doubtful etiological significance in colds. It is true that their incidence in the filtrates has been higher (86 per cent) in the positive experiments than in the negative (55 per cent). However these figures were reversed in our previous studies (2), and, as will be pointed out below, the organisms were present in equal proportions (100 per cent) in both our negative and positive human transmission experiments. That certain specific types, occurring in this extremely heterogeneous group of organisms may be found to possess etiological significance is not impossible. So far we have no evidence in support of this assumption.

In the course of the present work, 36 ape experiments have been completed. These may be divided into two groups. First, 28 experiments concerned directly with the testing of the hypothesis that colds may be caused by filtrable agents, and second, 8 miscellaneous experiments.

In the first group washings from individuals with colds were used in 20 instances and normal washings in 8. Of the former, 4 animals were excluded for statistical purposes for reasons given below. Of the remaining 16 animals, 7 contracted colds, 1 atypical; this represents a 44 per cent incidence of successful transmissions. The four experiments referred to above were not included because the inoculations

were made in animals just purchased which had recently suffered from respiratory infections. This has been done in the light of our present recognition of the post-cold insusceptibility referred to above. All eight of the control experiments, as noted above, were completely negative.

In the miscellaneous group, one was an experiment done with unfiltered nasal washings. It was positive. Two were ape to ape experiments, in which filtered ape washings were used. They were both positive. In order to determine the viability of the active agent three experiments were done with filtrates which had been stored in the ice-box 2 to 7 days. These were all negative. The remaining two experiments were done with living cultures of the filter-passing anaerobes obtained from a filtrate which had been used in a successful transmission experiment, these were both negative.

Finally, it is of importance to call attention to the following. On several occasions apes were in the quarantine room with others which were being used for transmission experiments. Several times when the latter had acquired experimental colds, the uninoculated animals contracted the infection in 3 or 4 days, presumably as a result of contact.

III. Transmission Experiments in Man

Having to our satisfaction worked out the above described effective quarantine technique for carrying out transmission experiments with apes, we considered it desirable to apply the same methods to man. We felt further that if we should utilize this rigorous isolation technique with man we could meet the usual criticism directed against human transmission experiments, as previously performed, without such strict quarantine.

Methods.—Human volunteers were secured through the employment bureau of the Hospital. Young adults who stated that they had had no colds or other respiratory infections in recent months and who had never had sinus complications or pneumonia were chosen. Reasonable intelligence and familiarity with the English language were also stressed in the selection of the volunteers.

The subjects were given a bath and were put into hospital clothes which had been autoclaved. They were placed immediately in an isolation room. They were not permitted to leave this room before the completion of the experiment.

The room had been previously cleaned with 2 per cent lysol solution and then aired for 24 hours. Everything in the room, including linen, toilet articles, occupational therapy material, with which the subjects whiled away their time, reading matter, etc., was carefully sterilized. Food entering the room was sterilized wherever possible.

The volunteers were attended by graduate nurses who were thoroughly versed in the principles of aseptic surgical technique and who always wore cap, gown, mask and rubber gloves upon entering the isolation room, and who used every precaution to prevent the possibility of entrance of infection from outside. Further, the visits of both nurses and workers were reduced to a minimum.

Upon entering quarantine the subjects were given a thorough physical examination, and a study, by daily culture, was begun of the flora of their noses and throats. In addition their sputum was tested by the mouse injection method for the presence of pneumococci. This was done as the finding of fixed types of these organisms was considered to be a contraindication to the use of the individual for inoculation.

As in the case of the apes, these human subjects were held for 5 days or more for preliminary observation, this being done to test the efficacy of the isolation and to exclude entrance during possible incubation of an upper respiratory infection.

At the termination of this period of observation, if the volunteer proved satisfactory, suitable colds were selected, washings obtained and filtered, and intranasal inoculation carried out in the manner described above for the ape experiments. For injection of the filtrate the subject was placed in the dorsal position for 2 to 3 minutes and then was turned over on his face for 1 minute.

Results.—Altogether 11 men were used in succession for this form of experiment. Two of the experiments were not completed. One because the attendant nurse contracted a cold just at the end of the preliminary period of observation and the other because the subject was found to be a carrier of Type III pneumococcus.

Of the 9 completed experiments 4 were positive. This represents a 44 per cent incidence of successful transmissions, a percentage incidence which is practically identical with that obtained in the work with anthropoids. A brief description of these colds will be given below. Before proceeding to do this, attention should be called to certain aspects of this type of experiment which are worthy of emphasis.

It is very easy for an individual who is being used for a transmission experiment to believe that he has a mild cold although objective evidence is extremely slight or absent. Where, as in the beginning of our work, volunteers believed that we were trying to produce colds, they were self-convinced occasionally that they were suffering from a

mild infection. This was much easier of belief as the filtrate in practically all the cases, negative and positive, causes some slight stuffiness of the nose, a little sneezing and occasionally slight headache.

Very early in the work we recognized this willingness of our subjects to oblige us and began taking measure to avoid this source of error. By various ruses, as nasal injections of sterile broth, collection of nasal washings for culture and equivocal statements, we were enabled to keep the subjects in ignorance. In certain instances, where the intelligence of the subjects was commensurate, we sought their direct cooperation.

A further help in ruling out error was dependence upon concrete objective findings. Such signs and symptoms as injection of the conjunctivae, profuse nasal discharge, muco-purulent post-nasal discharge, frank inflammation of the pharynx with lymphatic hyperplasia, continuous cough, etc., were considered indispensable for the interpretation of a result as positive.

Case H 4 is worthy of note as an example in this connection.

It was apparent very early that this individual was more or less unreliable and from the start it was possible to keep him in the dark regarding our procedure. He had inconspicuous symptoms after his test injection of sterile broth and no more striking results from the cold filtrate, until an assistant, on the second day after injection, inadvertently referred to his failure to contract a cold. That evening and night the subject reported severe symptomatology, including sneezing, cough, sore throat and stuffiness of the nose. The next morning he was told that he had been misinformed in regard to the nature of the filtrate and his symptoms subsided within the hour. It is important to note that there was an entire absence of objective pathological changes.

The 4 experimental colds that occurred in this group of 9 attempted transmissions, appeared in Cases 1, 3, 5 and 9 of the series. The incubation period in all cases was 24 hours or less. The first and third of these are shown in graphic fashion in Figs. 2 and 3.

Fig. 2 shows the 5 day period of preliminary observation, the time of inoculation and the appearance of symptoms after a little over 12 hours. The cold was a rather severe one, was much better on the fifth and sixth days (as shown on the chart) and then became considerably worse 2 days after release from quarantine. This finding will be referred to below. The bacteriological observations are shown on the lower half of the figure. At the onset and during the course of the cold

filtrate: $\downarrow\downarrow$

Case WHW	Date: Nov.	22	23	24	25	26	27	28	29	30	1	2
<u>Clinical</u> :-	Well	+	+	+	+	+						
	Stuffy nose						+					
	Sneezing						+	+				
	Coryza						++	++	++	+	+	
	Nasal Discharge						++	++	++	++	+	+
	Nasal obstruction						++	++	++	++		
	Cough						+	+	+			
	Sputum											
	Sore throat							+				
	Headache					+						
	Poor appetite						++	+				
	Malaise						+					
<u>Cultures</u> :	Non-hemol. Strep.						+	o	+	+		
	Diphtheroids						+	+	+	+		
	Staph. Albus	•	•	•	•	•	•	•	•	+		
Naso-pharyngeal-	Gram-neg. cocci	•	o	•	o	o	•	•	o	o		
	Non-hemol. strep.	o	•	o	•	•	o	•	•	•		
	Bacillus "X"						+	++		+		
	B. Pfeifferi	+	+					+		+		
	Staph. Albus	+	+					++				
	Pneumococcus					+	+					

FIG. 2. Experimental cold in man, showing clinical and bacteriological development. Symbols as in Fig. 1.

broth: \downarrow filtrate: \downarrow

Case LW.	Date	Jan:	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28
<u>Clinical</u> :-	Well		+	+	+	+	+	+	+	+							+
	Stuffy nose							+	+		±	+	+	+	+	±	
	Sneezing							+			±	+	+	+	±		
	Coryza																
	Nasal Discharge																
	Nasal obstruction												+	+	+	+	
	Cough																
	Sputum										+						
	Sore throat																
	Headache							+			+	++	++	++			
	Poor appetite										+	+	±				
	Malaise																
<u>Cultures</u> :	Diphtheroids		+				+	+	+		+	+	+	+			
	Staph. albus	•	•	•	•	•	•	•	•	•	•	•	•	•	•		
	Staph. aureus	o	o	o	o	o	+	o	o	o	o	o	o	o	o		
Naso-pharyngeal-	Gram-neg. cocci	o	o	o	o	o	o	o	o	o	o	o	o	o			
	Non-hemol. strep.	o	•	•	•	•	•	•	•	•	o	•	•	•			
	Bacillus "X"		++														
	B. Pfeifferi	+	+	+	+	+			+	+							
	Staph. albus										+		+				

FIG. 3. Experimental cold in man, showing results of preliminary sterile broth inoculation. Symbols as in Fig. 1.

there is no essential change in the characteristic flora of the nose and throat. The presence of pneumococci (IV) in the period of preliminary observation is probably without significance as we have observed this both in our normal studies and in the present experiments where results have been negative.

Fig. 3 shows a rather different type of cold. The symptoms of rhinitis and coryza were mild while the pharyngeal ones were quite conspicuous. The following is of much interest in this particular instance. While one of us was collecting the nasal washing for this case the patient coughed vigorously directly in worker's face. 2 days later, a few hours after the onset of the experimental cold this worker came down with an infection which was very similar, especially with respect to the comparatively negligible nasal symptoms. This figure shows also a typical result when sterile broth is injected as a preliminary step. Slight stuffiness of the nose and headache and a little sneezing appeared. These had completely subsided by the third day and the subject was quite free of symptoms when he received the cold filtrate. It will be noted again that there are no changes of importance in the bacterial flora.

Of the remaining two colds, one was a mild one in which sore throat, laryngitis and cough with moderate amounts of sputum, were conspicuous. The other was a simple uncomplicated mild cold. In these colds also no significant alterations in the bacterial flora, incident to the infections, were noted.

Of much interest to us has been the sequence of events in two of the three colds which we have been able to follow after release from isolation. In both these there was a definite exacerbation of the infection a day or two after leaving. This has been considered to be the probable result of protection from exposure to transient potential secondary invaders provided by the quarantine. The individual upon leaving again is accessible to such potential pathogens which become active upon the substrate of the experimental cold. In the light of the above, the volunteer who acquired the last experimental cold and who was the third of those whom we were able to follow was advised to stay away from crowds for a few days after being discharged. It is interesting but not necessarily significant that he had no recrudescence of his symptoms.

As noted above, filter-passing anaerobes were present in all cold filtrates used in these experiments irrespective of their outcome. Further, their type distribution was approximately the same in filtrates resulting in both positive and negative transmission experiments. This observation does not preclude the possibility that these organisms

play a part in the causation of colds, but it does constitute another link in the chain of evidence against this probability. Further studies directed toward more precise determination of the possible relationship of these organisms to colds are being pursued.*

DISCUSSION

Three points seem to be worthy of especial emphasis.

1. Chimpanzees would seem to be, from the foregoing, unusually satisfactory animals for use in the study of infection of the human upper respiratory tract. When they contract these infections, the clinical picture they present is strikingly similar to that observed in man. This taken together with the fact that their bacteriological flora is essentially like that of man and that they are closely related biologically to man suggests that their pathological and immunological response to bacteria pathogenic for the human upper respiratory tract may be sufficiently similar to make them the ideal experimental animal for this type of study. The tractability and cooperativeness of the chimpanzees makes them still more desirable. When well cared for they seem to keep in good health for long periods of time.

2. Colds may be transmitted to man and chimpanzee by intranasal inoculation of filtered nasal washings. These filtrates usually can be shown to contain anaerobic filter-passers of the type first described by Olitsky and Gates. However, our evidence so far points to the probably non-pathogenicity of these organisms for man. That specific types in this group may be shown later to play a part in causing colds has been pointed out above. If, however, as seems most probable at present, proof of this is not forthcoming, it follows that the active agent present in these filtrates, by means of which we have been able to transmit colds, is a true submicroscopic virus.

3. The sudden and plentiful appearance of pneumococci in the noses and throats of the chimpanzees in the course of colds has been most striking. The nature of this response is difficult of interpretation. There is little doubt that these organisms have been present

*Since this article was written the number of experimental colds transmitted by filtered nasal washings from which filter-passing Gram-negative anaerobes have been absent has increased so that it is now certain that colds can be transmitted by bacteria-free filtrates from the sources described.

before the infection, spontaneous or experimental, of the animal. They have been noted often in small numbers at various intervals in the throats of the animals and might well be shown to be regularly present by the mouse injection method. We have felt that their prominence may well be the result of multiplication and spread upon a substrate of primary injury due to the filtrable agent or that there may be some sort of activation of these potential pathogens by this agent. A like explanation may be offered for the spread to the nose of *B. pfeifferi* and hemolytic streptococci.

CONCLUSIONS

1. Chimpanzees are highly suitable animals for the experimental study of human upper respiratory infections.

2. Human colds have been successfully transmitted to apes and human volunteers in 44 per cent of instances tried by means of filtered nasal washings obtained from colds.

3. Certain types of infectious colds are caused by a filtrable agent.

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STUDIES IN THE SEROLOGY OF SYPHILIS

I. THE MECHANISM OF THE FLOCCULATION REACTIONS

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In alcoholic solution, the normal tissue lipoids used as "antigen" in the serum-diagnosis of syphilis are probably molecularly dispersed. Upon dilution with water, however, there is a marked aggregation of the lipid molecules. The originally transparent solution becomes opalescent, gives a Tyndall phenomenon, and by darkfield examination, innumerable highly refractile lipid particles in active Brownian motion can be seen. Their average size, the number visible, and therefore the degree of opalescence, depend upon the method of admixture of the two liquids (Sachs and Rondoni, 1909).

If, to a diluted antigen in which all these microscopically visible particles are discrete, one adds syphilitic serum, there is, as Jacobsthal (1911) observed, a rapid clumping of the apparently unchanged lipid micellae into coherent aggregates. Depending upon the concentration of the reagents, the temperature, time of incubation, etc., these aggregates may or may not exceed the limits of colloidal stability to form the optically visible "precipitate" first noted by Michaelis (1907). As he predicted, this precipitation phenomenon has found wide-spread diagnostic application. The Sachs-Georgi and Kahn precipitation tests, the Vernes flocculation reaction, the Murata test, the Sigma reaction, the Meinicke-Trübungs, Meinicke-Klärungs, and Müller-Ballungs reactions, and the Hinton agglutination reaction are all basically the same. The lipid antigen, modified by the addition of tolu balsam, cholesterol, glycerol, NaCl, etc., forms a more or less stable "solution" (suspension) in normal serum; but in syphilitic serum there is a visible aggregation.

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The causes of this aggregation and the optimal conditions for its production are the subjects of the present paper.

I. Surface Properties of the Lipoid Antigen

Colloids in general are divided more or less arbitrarily into two heterogeneous groups, between which there is no sharp distinction, but which, at the extremes, are quite characteristic (Svedberg, 1924): hydrophobic, with no affinity to water, and therefore readily flocculated by electrolytes (gold sol; Fe_2O_3 sol), and hydrophilic (proteins; glycogen).

The lipid antigen in alcoholic solution is probably molecularly dispersed. Diluted with water, however, the molecules aggregate to form a colloidal suspension, the properties of which are intermediate between those of the hydrophobic and hydrophilic classes. The colloiddally dispersed lipid is amphoteric, flocculating at its isoelectric point, pH 1.9 (Fig. 3), but relatively stable at more alkaline reactions. Thus, at pH 6.0 (Figs. 1 and 2) it requires about 1 N concentration of univalent cations to cause aggregation of the dilute sol (0.04 per cent), and, as usually found with negatively charged particles, about 1/40 as much of bivalent cations (BaCl_2). The critical potential, the minimum compatible with stability, is 2 to 5 millivolts, much lower than that of most hydrophobic colloids, and strongly suggesting some other factor making for stability, analogous to hydrophilic colloids.

Protocol 1

The procedure followed in the preparation of a concentrated antigen was in essential details that used by Kahn (1925). 250 gm. of dehydrated powdered beef heart were shaken for 10 minutes with 1000 cc. ether, the filtrate discarded and the process repeated twice again with the residue. The dry powder remaining was weighed, and extracted for 3 days with 95 per cent ethyl alcohol (5 cc. per gram). Cholesterin is added to the pale yellow filtrate to any desired concentration.

5 cc. of antigen containing 1.5 per cent of beef heart lipid and 0.2 per cent of added cholesterin were shaken with 5 cc. of NaCl N/7, and the suspension centrifuged. The sediment is shaken up in 10 cc. H_2O , forming a suspension containing approximately 0.7 per cent lipid, varying quantities of which are dropped into electrolytes of various concentration. The pH is kept within an approximate range by phosphate buffers (final concentration M/300), the exact value being determined potentiometrically. The total volume is brought up to 4 cc. with H_2O , the tubes shaken, and flocculation read after 24 hours at room temperature. The data are summarized in Figs. 1 and 2.

One other point should be mentioned here, which will be referred to again in another connection. The concentration of electrolytes necessary to produce flocculation (coagulation value) is not a fixed quantity, an intrinsic property of the given colloid, but varies markedly with the concentration of the sol. As seen in Table I and Fig. 2, the more particles per unit volume, the less stable is the suspension. A smaller concentration of electrolyte suffices to produce aggregation; and the

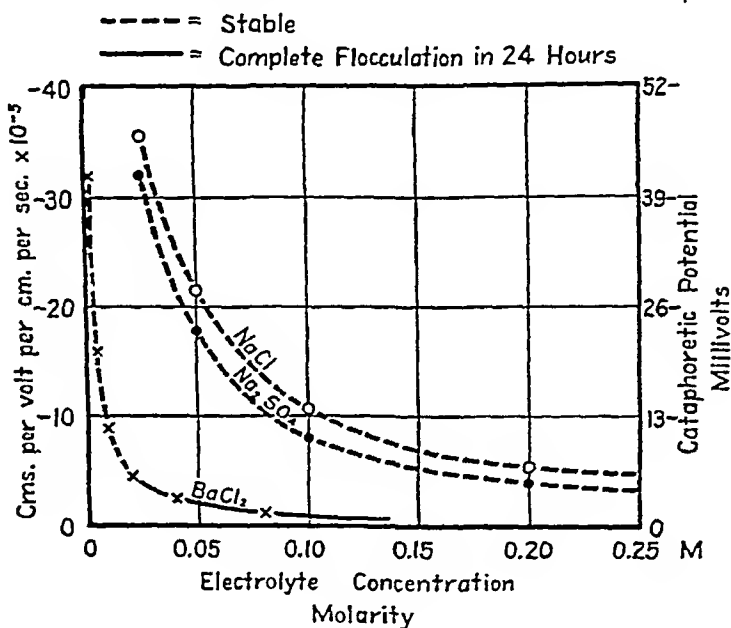


FIG. 1. Effect of electrolytes upon electrokinetic potential and stability of lipid sol (0.04 per cent) at pH 6.0.

surface charge necessary to keep the particles dispersed and thus ensure stability becomes progressively higher. The theoretical considerations involved are discussed by Freundlich (1922).

II. The Effect of Normal Serum upon the Lipoid Antigen

Serum proteins in solution are, of course, highly hydrophilic. When adsorbed at the surface of a heterogeneous phase, however, they may act in either of two diametrically opposed senses, depending upon the

nature of the absorbent. They may retain their hydrophilic properties and form a protective film around a hydrophobic particle, preventing its flocculation by electrolytes; or they may become denatured when adsorbed, lose their hydrophilic properties, and become instead water-insoluble, hydrophobic; they then sensitize the adsorbing particle, making it even more susceptible to flocculation by electrolytes.

The nature of this denaturation is not clearly known; full discussions are to be found elsewhere (Freundlich, 1922, 1924). The point of interest is that serum globulin, when adsorbed by a colloiddally dispersed heterogeneous phase, may form either a protective or sensitizing

TABLE I

Effect of Increasing Concentration of Lipoid Sol (Antigen) upon Its Stability and Coagulation Value (Fig. 1)

NaCl, normality.....	0.1	0.2	0.4	0.8	1.6
Concentration of lipid in sol:					
0.002 per cent.....	0	0	0	0	2
0.008 per cent.....	0	0	0	1	2
0.032 per cent.....	0	0	0	3	4
0.128 per cent.....	1	1	3	4	4
0.512 per cent.....	3	3	4	4	4

Figures in body of table indicate agglutination after 24 hours.

4—complete, with clear fluid.

3, 2, 1—degrees of aggregation.

0—no optically visible aggregates.

film, depending upon the nature of the adsorbent, the determining factors being unknown.

When the lipid antigen is added to normal serum, one can demonstrate a remarkably avid adsorption of the serum protein by the particles of the colloiddally dispersed lipid.

Protocol 2

5 cc. of antigen were shaken with 5 cc. of NaCl N/7, the suspension centrifuged to remove the alcohol, and resuspended to 10 cc. in H₂O. Series of tubes were set up similar to those outlined in Tables II and III, differing only in the concentration of normal, i.e., Wassermann and Kahn negative serum. Cataphoretic velocities were measured in the simple Michaelis (1926) chamber, care being taken to make

readings one-fifth of the distance from the top and bottom of the chamber to avoid endosmotic currents, and in both directions to counteract drifts due to gravity. The values given for the velocity in the tables and in the figures were obtained by measuring the time required for the particle to pass between two points on an ocular-micrometer scale. The absolute values of this velocity of the electrokinetic potential it implies (see note to Tables II and III) have only a qualitative significance, the important factor being the isoelectric point, the hydrogen ion concentration at which the charge on the particle changes sign (equal ionization as acid and base), and at which there is therefore no movement in an electrical field.

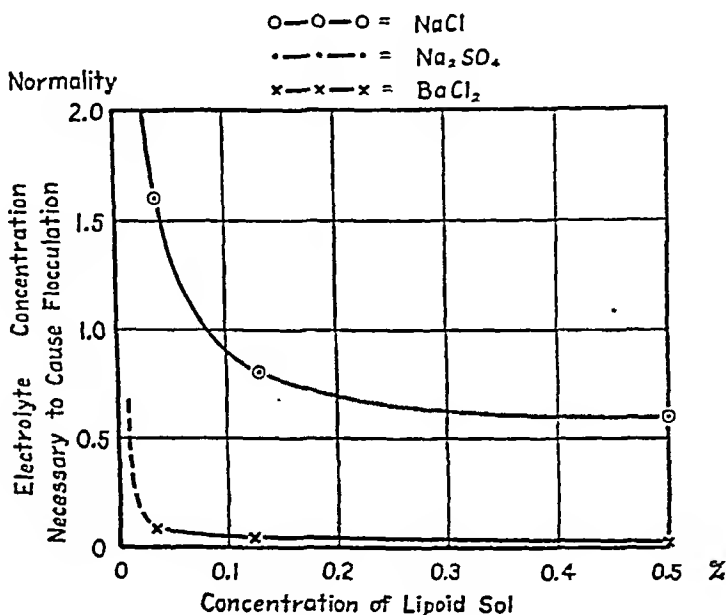


FIG. 2. Effect of concentration of lipid upon its stability (pH 7.4).

Hydrogen ion concentrations were determined with the quinhydrone electrode, the error being ± 0.05 pH.

The solid lines in all the figures indicate zones of flocculation, uniformly corresponding to zones of minimal potential; + signs indicate a particle positively charged towards the water, migrating to the cathode in an electrical field, while — signs imply a negative electrokinetic potential.

A. Isoelectric Point.—As little as 1:4000 serum (*i.e.*, 1:50,000 protein) suffices to alter the surface properties of the lipid particles significantly. The zone of optimal flocculation, coinciding with the

cataphoretic isoelectric point, shifts from $\text{pH } 1.9 \pm$, that of the lipid antigen particles, towards a more alkaline reaction, the degree of shift depending upon the concentration of serum protein. It is significant that the maximum change is to $\text{pH } 4.9$, midway between the isoelectric points of serum albumen and serum globulin; the particle is then completely covered with protein, and has the same surface properties. With less serum, however, the amount of protein adsorbed does not suffice to cover the cell; the charge and the isoelec-

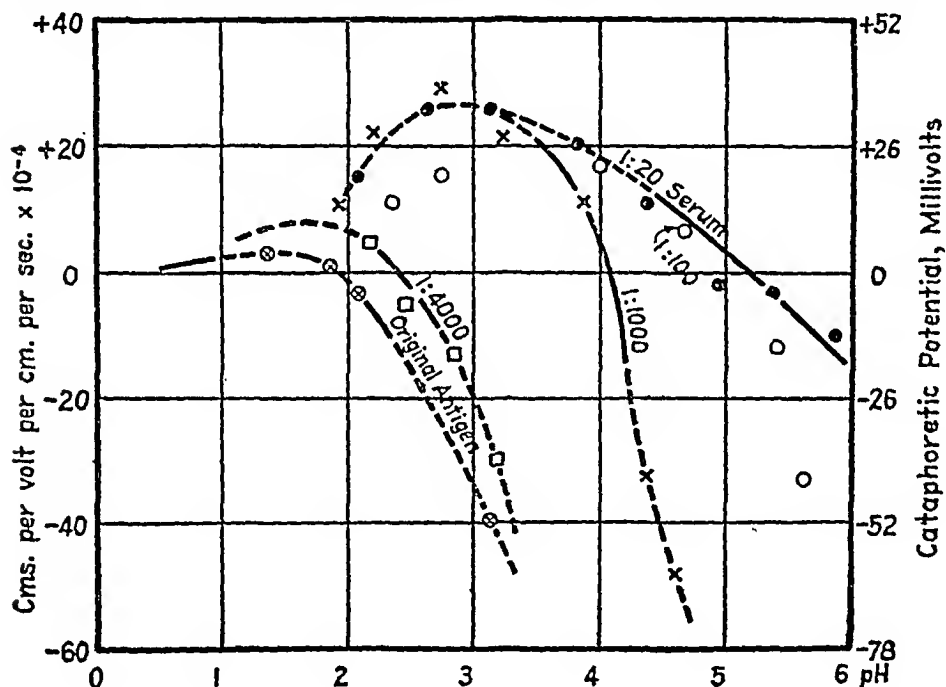


FIG. 3. Effect of normal serum upon cataphoresis of the antigen particles.

tric point, determined as they are by a mosaic of protein and lipid, therefore have some intermediate value, the exact value of which depends upon the proportions of the two types of surface. This is more clearly shown in Fig. 4.

In addition to affecting the isoelectric point, this adsorbed normal serum protein changes the surface charge (and cataphoretic velocity) of the antigen at all reactions, corresponding to a change from a surface of lecithin to one of serum protein, the degree of shift again depending upon the extent of the adsorbed film.

TABLE II

Effect of pH upon Surface Properties of the Lipoid Antigen in Absence of Serum
(Fig. 3, Curve O—O)

HCl, N/1, cc.....	3.8	1.0	0.25	0.062	0.05				
" N/100, cc.....						1.6	0.4	0.1	0.025
H ₂ O, cc.....	0	2.8	3.55	3.75	3.75	2.2	3.4	3.7	3.8
Antigen suspension, cc...	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2
pH.....	<1	<1	1.35	1.86	2.0	2.51	3.14	3.8	4.56
Cataphoretic velocity cm. per (volt per cm.) per sec. $\times 10^{-5}$	<1	+1	+3.6	+1.4	-3	-16	-40	>-80	>-80
Surface charge* milli- volts.....	<+1	+1.3	+4.7	+1.8	-4	-20	-52	>-100	>-100
Agglutination in 2 hours.	\pm	0	+	++++	+++	0	0	0	0

TABLE III

Effect of pH upon Surface Properties of the Lipoid Antigen in 1:1000 Normal Serum
(Fig. 3, x - x Curve)

HCl, N 1/10, cc.....	1.28	0.64							
HCl, N 1/100, cc.....			3.2	0.8	0.4	0.2	0.1		
HCl N 1/1000, cc.....								0.8	0.4
H ₂ O.....	3.2	3.86	1.3	3.7	4.1	4.3	4.4	3.7	4.1
Antigen sus- pension + 1:100 serum..	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5
pH.....	1.64	1.93	2.21	2.88	3.23	3.88	4.38	4.6	5.14
Cataphoretic velocity cm. per volt per cm. sec. \times 10^{-5}	+2.5	+10	+22.4	+29.1	+21.3	+10.3	-32	-48	>-80
Electrokinetic potential,* millivolts....	+3	+13	+27	+38	+28	+11.4	-39	-62.4	>-100
Macroscopic agglutina- tion in 2 hours	0	0	0	0	0	\pm	+++	\pm	0

+ = Positive charge; migration to cathode.

- = Negative charge; migration to anode.

$$* \text{ Electrokinetic potential (electrostatic units) } = \frac{4 \pi \eta}{KX} u$$

$$= \frac{4 \pi \times \text{coefficient of viscosity}}{\text{dielectric constant} \times \text{potential gradient (E.S.U.)}} \times \text{velocity}$$

$$\text{Potential (volts)} = 300 \left[\frac{12.57 \times 0.0093 (23^\circ\text{C.})}{80} \right] \times \text{velocity per volt per cm. per sec.} \times 300$$

$$= 13 \times \text{velocity per volt per cm. per sec.}$$

Protocol 3

To each of a series of tubes were added 0.2 cc. of phosphate buffer (M/15) at pH 7.4, varying quantities of serum, and H₂O to 4 cc. A similar control series was prepared, containing varying quantities of NaCl 0.85 per cent instead of serum as in the first series. A washed suspension of antigen was added to each tube and cataphoretic velocity determined. The experimental data are summarized in Fig. 5.

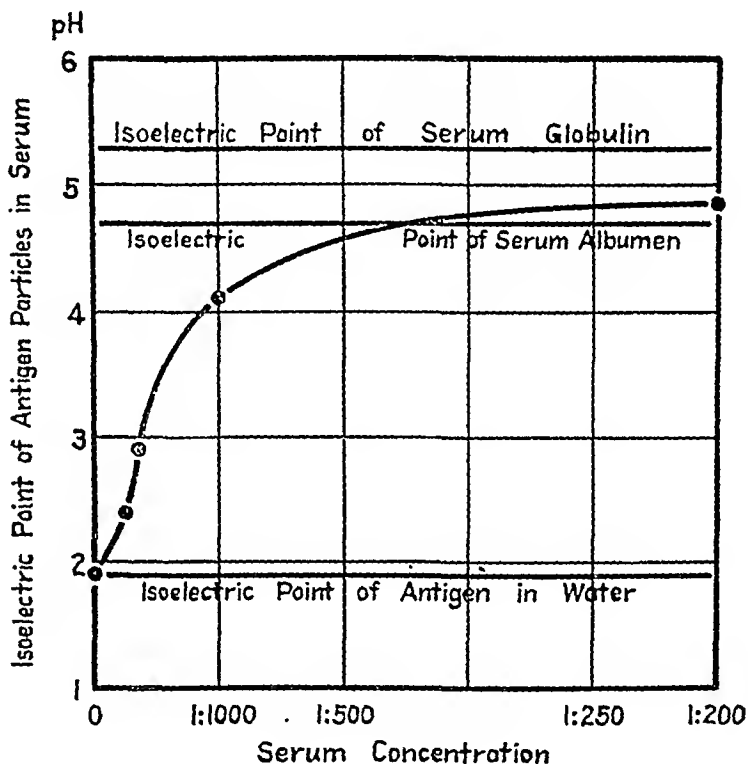


FIG. 4. Isoelectric point of antigen particles in serum is determined by adsorbed serum protein.

B. Flocculation.—Despite the fact that, as just shown, serum protein is strongly adsorbed by the lipoid antigen, there is no significant change in its stability, as determined by its tendency to flocculate. True, the isoelectric point, and therefore the range of optimum flocculation, shift towards that of the adsorbed protein: but away from this reaction the suspension is even more stable than before the addition of serum. The adsorbed protein remains hydrophilic, and acts as a film of pro-

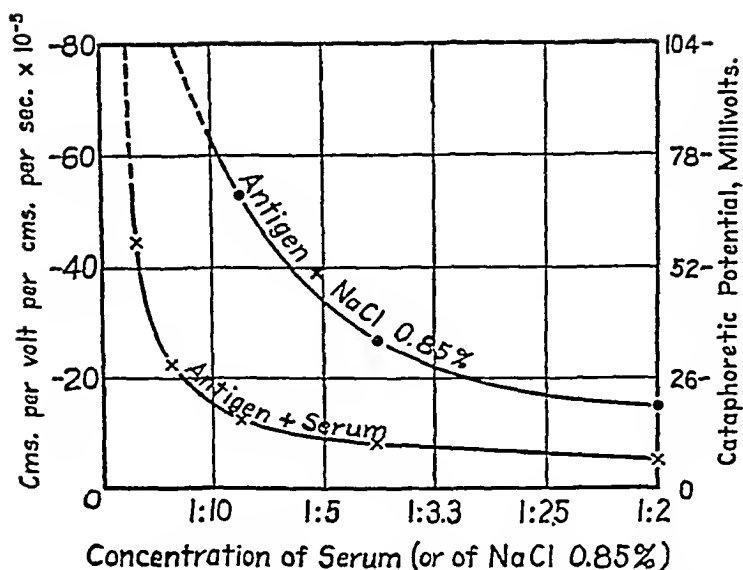


FIG. 5. Effect of normal serum upon surface properties of antigen at pH 7.4.

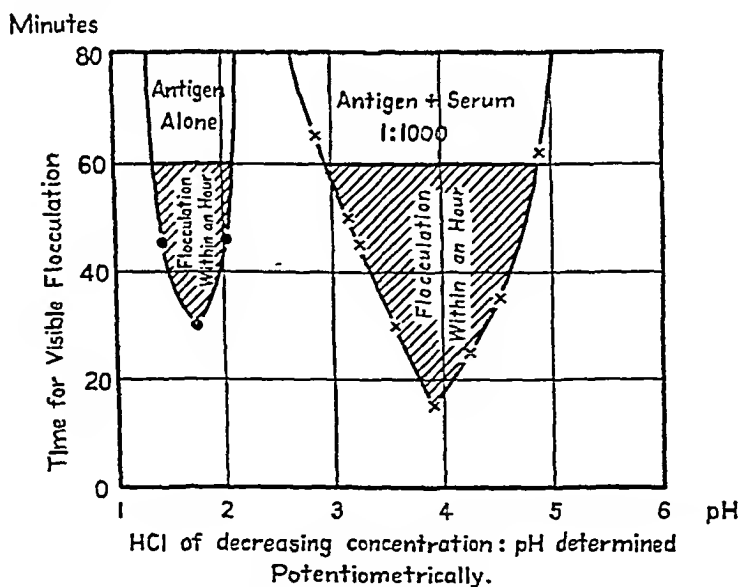


FIG. 6. Effect of normal serum upon flocculating properties of antigen.

fective colloid, preventing even the normal flocculation of antigen at its own isoelectric point (Fig. 6): while at serum reaction (pH 7.4) just as much electrolyte is required to cause flocculation in the presence of serum as in its complete absence.

Possibly, at high electrolyte concentration (e.g., $> M/2$) the adsorption of normal serum protein is prevented, allowing the flocculation of the uncovered lipid particle by electrolytes. At any rate, the critical potential and the coagulation value, and therefore stability, are not affected by normal serum.

To summarize, normal serum protein is strongly adsorbed by the lipid antigen,* forming a protective film of hydrophilic protein around the constituent particles, and adding to their stability away from their isoelectric point.

III. Properties of the Lipoid in Syphilitic Serum

When the antigen is placed in syphilitic serum, there is, of course, the same non-specific adsorption of normal serum protein. In addition, however, it combines with a specific component, so-called "reagin," with a striking change in its surface properties.

A. Composition of the Lipoid-Reagin Precipitate.—

Like true antibodies, this "reagin" is always associated with the globulin fraction of serum protein (Kapsenberg, 1924) (Sahlmann, 1922) (Gloor and Klinger, 1920). Such evidence as there is to the contrary (Felke, 1921; Skrop, 1923) has been vigorously discredited by Stern (1923). *A priori*, then, the precipitate should consist of the antigen plus the reagin-globulin with which it has combined. On this point, however, there is conflicting evidence.

* This adsorption of normal serum protein at all reactions is in itself the strongest evidence against the theory (Epstein and Paul, 1922) that syphilitic reagin is a positively charged colloid, which combines with the negatively charged lipid particle, with mutual discharge and precipitation. There is no experimental evidence for this theory (Bauer and Nyiri, 1921), (Stern, 1923, 1924); moreover, it fails to explain why flocculation is not obtained when syphilitic serum is added to any negatively charged suspension. The fact that normal protein is adsorbed by lipid even when both have the same charge (both negative at serum reaction) shows that one can not predicate opposite charges as the cause for the combination of lipid with reagin, and their subsequent flocculation.

Jacobsthal (1911) considered the aggregates to be lipoidal, as did Niederhoff (1921), and Epstein and Paul (1921, 1922). Meinicke (1919), on the other hand, misled by his two-phase reaction, considered the precipitate to be entirely proteid, disregarding the fact that microscopically one can see the aggregation of the lipid particles. Following Georgi (1919), who showed that the precipitate is not completely soluble in alcohol and ether, the exact analyses of Klostermann and Weisbach (1921) indicate that about 20 per cent of the precipitate can not be redissolved in alcohol and ether, and that of the insoluble residue at least one half (10 per cent of the total) is serum globulin; while Scheer (1921) considers the globulin to constitute 36 to 60 per cent of the total precipitate.

The following qualitative experiment shows clearly that the precipitate does consist chiefly (80 to 90 per cent) of the antigen lipid, plus some specific component of syphilitic serum with which it has combined, a substance known to be associated with the globulin fraction of serum, giving the routine tests for protein and which can therefore be called reagin-globulin.

Protocol 4

Preparation of Large Quantities of Antigen-Reagin Precipitate.—5 cc. of the alcoholic extract containing 0.6 per cent cholesterin were mixed with 5 cc. of NaCl N/7 and the white suspension thus obtained shaken for a few minutes with 100 cc. of strongly positive syphilitic serum. After $\frac{1}{2}$ hour at 56°C. and 24 hours at ice box temperature the suspension was diluted several times with saline in order to facilitate sedimentation, and centrifuged at 2500 r.p.m. for 30 minutes. The sediment was washed twice in 250 cc. NaCl N/7, once in H₂O and finally centrifuged in a graduated tube until its volume remained constant.

Qualitative Analysis of the Precipitate.—A known volume of sediment was extracted with 15 cc. of alcohol, then with 15 cc. of ether, and finally again with 15 cc. of alcohol. The residual precipitate was centrifuged in a hematocrit tube until its volume remained constant. As seen in Table IV, from 10 to 20 per cent by volume of the antigen-reagin precipitate is insoluble in alcohol and ether.

The combined alcohol and ether extractions, representing 80 to 90 per cent of the total precipitate, when evaporated to dryness and redissolved in alcohol, form as efficient an antigen as the original cholesterinized extract; the antigenic lipoids are carried down unchanged in the precipitate. The 10 to 20 per cent residue is insoluble in water and saline; but since it contains N, and give the routine tests for protein (biuret, Millon, xanthoproteic), it must consist in part, if not wholly, of denatured serum protein.

A quantitative gravimetric analysis of the precipitate has no real

significance. The proportion of alcohol-soluble lipoids to serum globulin will depend upon the reagin-titre of the serum used and the relative amounts of serum and antigen; moreover, such large percentages of protein as found by Scheer represent non-specific normal protein, incompletely removed by washing.

Protocol 5

8 cc. of saline were shaken with 8 cc. of cholesterinized antigen, the resulting suspension centrifuged, resuspended to 15 cc. and added with shaking to 100 cc. of strongly positive syphilitic serum. After 24 hours in the ice box the mixture was diluted with 300 cc. NaCl, N/7 (pH 7.4) and centrifuged. The sediment,

TABLE IV
Composition of Antigen-Reagin Precipitate

Antigen-reagin precipitate	Residue after extraction with alcohol and ether	Alcohol-ether soluble (volumetric)
cc.	cc.	per cent
0.2	0.038	81
0.5	0.05	90
2.0	0.28	86

taken up in 50 cc. NaCl, is Suspension 1, containing at most 1:50 parts of serum. After 2 hours at room temperature, the suspension was again centrifuged, and again made up to 50 cc. (Suspension 2, <1:2500 serum). The final suspension obtained after 4 such washings represents at least 1:10⁷ serum dilution, verified by the N content of the supernatant fluid.

The isoelectric point of each suspension was determined cataphoretically and by the zone of optimal flocculation. The experimental data are summarized in Fig. 7, Table V corresponding to Curve 4 of the figure.

B. Effect of Washing.—

Normal serum alters the surface properties of the lipid particles by virtue of the protein adsorbed. The following experiment shows that in the lipid-reagin precipitate also, the protein detected chemically is present as a film around the lipid particles, but more or less irreversibly adsorbed, and thus similar to antibody protein bound by a specific antigen.

Clearly, the protein taken up by the antigen from syphilitic serum is present as an incomplete film around the individual lipid particles, not removed by washing. The isoelectric range of Suspension 4,

washed so thoroughly that it contains $<10^{-7}$ parts of free serum, is pH $3.4 \pm$, the same as antigen suspended in 1:2000 serum, instead of the normal value of pH 1.9. Once the normal serum has been removed (1 to 2 washings), repeated further washing makes no more change in the surface properties than is indicated by the shaded zone of Fig. 7. Assuming an approximately equal ionization of the lipid and protein surfaces per unit area, the shift in isoelectric point to pH 3.4 implies a lipid particle roughly one-half covered with protein.

TABLE V

Effect of pH upon Surface Properties of the Lipoid-Reagin Precipitate, Washed until Supernatant Is Practically N Free (Fig. 7, Curve □—□)

HCl, N/10, cc.....	1	0.25					
" N/100, cc.....			1.25	0.62	0.31	0.16	0.08
H ₂ O, cc.....	2.8	3.55	2.55	3.2	3.5	3.65	3.70
Precipitate suspension, cc.....	0.2	0.2	0.2	0.2	0.2	0.2	0.2
pH.....	1.64	2.2	2.52	2.81	3.13	3.56	4.22
Cataphoretic velocity cm. per volt per cm. per sec. $\times 10^{-5}$...	+1.3	+10	16	+12	+10.8	-13.3	-40
Electrokinetic potential,* milli- volts.....	+1.7	+13	+20.8	+15.6	+14	-17.3	-52.4
Agglutination in 2 hours.....	+	0	0	0	++++	++++	0

- = Negative charge; migration to anode.

+ = Positive charge; migration to cathode.

* See note at bottom of Table III.

Antigen therefore combines irreversibly with a specific protein of syphilitic serum. It is significant that Otto and Winkler (1922) arrived at the same conclusion by an entirely different line of investigation. They found that the washed lipid-reagin precipitate sensitized 80 per cent of thirty-two guinea pigs to the subsequent injection of human serum, proving the presence of serum protein in the precipitate; while of the controls, nineteen guinea pigs injected with the lipoids washed after immersion in negative serum, only 20 per cent showed slight sensitivity to human serum, subsequently injected, and none died of anaphylactic shock.*

* Similarly, agglutinated bacteria (Braun, 1909) or red cells (Altmann, 1912) sensitize an animal to the subsequent injection of serum of the same species as the antiserum.

There is therefore a striking analogy to the so-called specific immune reactions. Bacteria, red cells, and lipoid antigen all adsorb normal, as well as antibody protein: but while the normal serum protein, remaining hydrophilic, is readily removed by washing, the antibody globulin is firmly bound, and has been shown (chemically, immunologically, and by surface properties) to form a more or less irreversible denatured film around the antigen (Eagle, 1929, 2, 3; 1930).

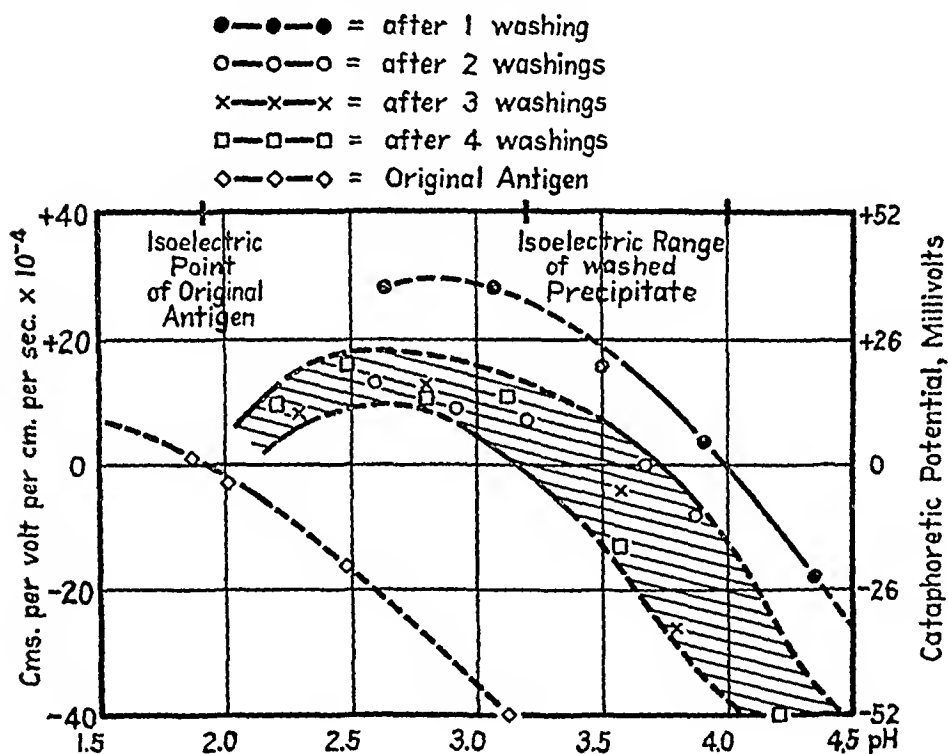


FIG. 7. Effect of washing upon the surface properties of the lipid-reagin precipitate.

It has already been shown (Eagle, 1930) that the flocculation of bacteria or of red cells by the antiserum is due to this film of antibody protein, denatured by its combination with the antigen, and therefore sensitizing the antigen-antibody complex to flocculation by electrolytes. The obvious implication is that the flocculation of the lipid syphilitic serum is an exactly similar process, an hypothesis which is confirmed in the following sections.

C. Flocculating Properties of the Lipoid-Reagin Complex.—

Normal serum protein affected only the isoelectric point and the cataphoretic potential of the antigen, without changing its critical potential. The adsorbed film retained its hydrophilic properties, and the suspension therefore remained stable up to about 1 M NaCl or 0.02 M BaCl₂, the exact coagulation value depending upon the concentration of lipid.

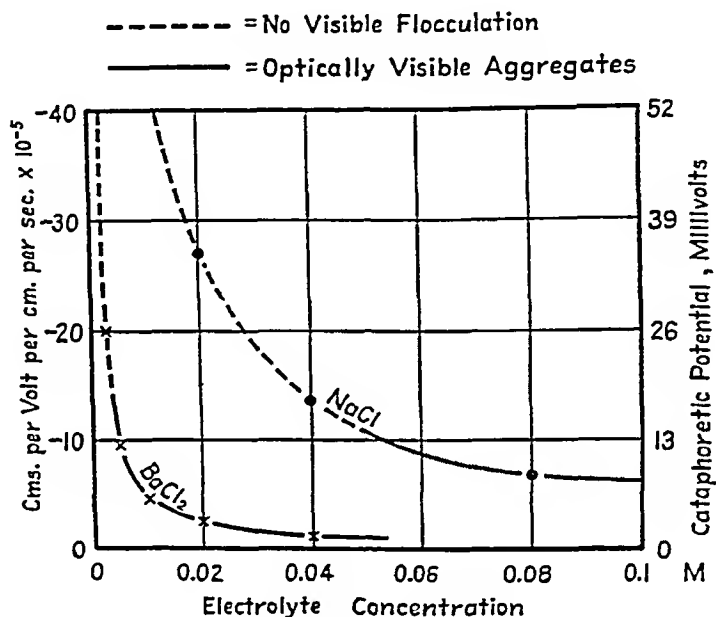


FIG. 8. Coagulation value of lipoid-reagin precipitate.

In marked contrast, the lipoid-reagin complex is flocculated at any hydrogen ion concentration by traces of electrolyte. The reagin with which the antigen particles combine not only changes their isoelectric point and lowers their cataphoretic potential exactly as does normal serum protein, but in addition, raises their critical potential from 1 to 5 millivolts to 10 to 15 millivolts (Figs. 8 and 9), an increase in cohesive tendency of approximately fivefold.

It is therefore more than a coincidence that such dissimilar particles as bacteria, red cells, protein micellae, and beef heart lecithin should

have exactly the same isoelectric point, cataphoretic potential, and in particular, the same critical potential, after sensitization with the homologous antiserum (antibacterial, hemolytic, precipitating and syphilitic respectively). In all these antigen-antibody complexes, the surface of the originally dissimilar antigen particles has been covered with an identical film of specific globulin, in some way altered by its combination so as to lose its affinity to the aqueous phase.

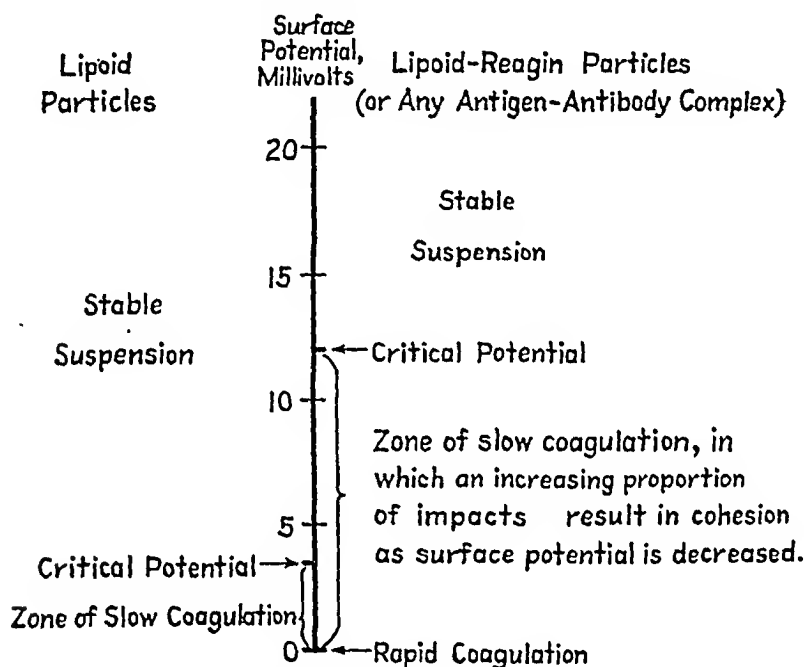


FIG. 9. Contrast between surface properties of antigen particles before and after combination with reagin.

At serum reaction, it ionizes as Na^+ globulinate $^-$, negatively charged because of the greater mobility of the inorganic ion. In the absence of electrolytes, this mutually repellent surface charge suffices to prevent cohesion of the particles as they approach during Brownian movement: the suspension is stable. Upon the addition of electrolytes the charge is depressed (Fig. 8). So soon as it falls below the critical value for denatured globulin (10 to 15 millivolts), the globulin-coated particles can approach within their radius of attraction, and aggregates form which sediment. The discharging ion is the one opposite in

charge to the particle, the cation; and salts with bivalent cations, which are 20 to 40 times as effective in discharging, are correspondingly more active in causing, flocculation. Thus (Fig. 8), the coagulation value for NaCl (or Na_2SO_4) is 1/20 to 1/15 M, for BaCl M/400 to M/200; quantities to be contrasted with the corresponding values for the original antigen suspension, before it acquired its sensitizing cohesive globulin film.

At first sight, it seems paradoxical that normal serum protein, when adsorbed, forms a protective hydrophilic film, while the reagin-protein serves to decrease the stability of the lipoid with which it has combined, by forming a sensitizing film of denatured hydrophobic protein. There are, however, many examples of serum protein serving in both capacities. Aside from the specific reactions already cited there is the action of protein-containing spinal fluid upon a colloidal solution of mastic or gold: in large quantities, the fluid may protect the sol against flocculation (hydrophilic film of protein), in smaller quantities, sensitize; there is the prozone in specific agglutination; the sensitizing action of globulin on Fe_2O_3 sols, and its protective action on dyes, etc. Moreover, the forces making for the loose reversible adsorption of normal serum protein are quite different from the specific affinity between the lipoid and the reagin globulin, as shown by the irreversibility of the latter combination, a difference further illustrated in the following section.

D. Complement-Fixing Properties of the Lipoid-Reagin Precipitate.—

The loss of its affinity for water is not the only change produced in a specific antibody globulin when it combines with antigen (bacteria, red cells, or dissolved protein). In addition to becoming denatured, and thus causing the flocculation of the antigen with which it has combined, it develops a marked avidity for complement (the hemolytic substance present in fresh serum), adsorbing it irreversibly (complement fixation). When the antigen-antibody complex is heated to 100° for a few seconds, this remarkable property is completely destroyed, presumably because of the heat-coagulation of the active film of denatured antibody (Eagle, 1929, 2).

If the mechanism of the precipitation of lipoid antigen by syphilitic serum is, as outlined in the preceding sections, exactly analogous to these specific antigen-antibody reactions, the precipitate formed should

possess the same complement-fixing properties as agglutinated red cells, bacteria, or a protein-antiprotein precipitate.

Antigen alone does not fix complement; nor does an adsorbed film of normal serum protein endow it with this property. As already shown by Wassermann (1921), and Ravenel and Dulaney (1925), however, the precipitate obtained with syphilitic serum fixes complement powerfully (Table VI, Row 1).

Protocol 6

x cc. of the washed lipid-reagin precipitate were incubated with 0.4 cc. of 1:10 complement for $\frac{1}{2}$ hour at 37°, and residual complement determined by a method already described (Eagle, 1929, 1). An individual experiment is given in detail.

Precipitate suspension, cc.....	0.4	0.2	0.1	0.05	0.025	0.0125	0.006
Time for hemolysis of sensitized cells added after $\frac{1}{2}$ hour, in seconds....	>1800	>1800	>1800	360	135	105	90
Per cent complement fixed.....	>90	>90	>90	70	40	15	<10

0.4 cc. of complement causes hemolysis in 90 seconds

0.2 " " " " " " 150 "

0.1 " " " " " " 480 "

0.05 " " " " " " 900 "

A series of experiments are summarized in Table VI, omitting similar technical details.

Even more interesting are the results in the second part of the table. A film of normal serum protein does not interfere with the specific reaction between the antigen particles and subsequently added syphilitic serum (reagin): the normal protein is only loosely bound and does not obscure the specific reacting groups of the lipid. The lipid-reagin precipitate, however, incubated with very strongly positive syphilitic serum, does not give any further reaction; the first incubation has covered the reacting groups of the lipid with closely adherent reagin-globulin, so firmly bound as not to allow combination with more reagin (Row 3). Heating at 100° for a few seconds coagulates and destroys this complement-fixing film of protein without affecting the underlying lipid. Their reacting groups once again free, the antigen particles give powerful fixation with the same syphilitic serum which was previously ineffective (Row 6).

The reagin film therefore differs from adsorbed normal protein not

only in its water-insolubility, and tendency to flocculate; but also in its ability to fix (adsorb) complement. Moreover, unlike normal protein, it attaches so firmly to the specific groups of the lipoid as to prevent any

TABLE VI
Complement-Fixing Properties of the Lipoid-Reagin Precipitate

Precipitate suspension, cc.....	0.1	0.05	0.025	0.0125	0.006	0.003	0.0015	
Per cent complement fixed by x cc. of lip- oid-reagin precipitate suspension.....	>90	60	35	20	10	<10	<10	
Per cent complement fixed by x cc. sus- pension + 0.4 cc. of 1:40 antigen.....	>90	60	35	20	10	<10	<10	No free reagin
Per cent complement fixed by x cc. sus- pension, + 0.1 cc. very strongly posi- tive serum.....	>90	70	40	25	10	<10	<10	No free antigen (at most, minimal quantities)
Per cent complement fixed by x cc. of sus- pension, heated to 100°C. for 1 minute..	0	0	0	0	0	0	0	Adsorbing reagin film destroyed by heat
Per cent complement fixed by x cc. heated suspension, + 0.4 cc. antigen 1:40.....	0	0	0	0	0	0	0	No free reagin
Per cent complement fixed by x cc. heated suspension, + 0.1 cc. very strongly positive syphilitic serum	>90	>90	>90	>90	>90	75	40	Free antigen

further combination with additional reagin. For lack of a better term, we may call this group of properties, induced in all antibodies by their combination with antigen, denaturation, remembering it to be quite distinct from the coagulation of a protein by heat.

IV. SUMMARY AND DISCUSSION

A. The lipid antigen used in the serum diagnosis of syphilis, when colloiddally dispersed in water, forms a relatively stable amphoteric suspension with predominantly hydrophilic properties. Although the colloidal particles flocculate at their isoelectric point (pH 1.9), in more alkaline reaction the negative surface potential prevents their cohesion and must be depressed to 1 to 5 millivolts before visible flocculation is obtained, indicating a very slight affinity between the colloidal particles. The amount of electrolyte necessary to depress this surface charge below its critical value decreases somewhat with increasing concentration of the sol, but is uniformly large: in a suspension containing 0.04 per cent lipid, 1 M univalent and 1/40 M bivalent cation are the coagulation values.

B. In normal serum, hydrophilic protein is adsorbed, forming a protective film around the individual lipid particles, with a corresponding change in the cataphoretic potential and the isoelectric point towards those of serum protein, the degree of shift depending upon the extent of the adsorbed film. The critical potential, however, is not affected, and the lipid remains as stable away from its isoelectric point as in the absence of serum. The water-soluble film of unchanged protein is readily removed by washing, and does not prevent the subsequent combination of the underlying lipid with the specific component of syphilitic serum.

C. When the lipid antigen is added to syphilitic serum, in addition to this loose adsorption of normal protein it combines more or less irreversibly with a specifically altered fraction of the serum globulin (reagin), demonstrable in the washed precipitate both chemically and by sensitization experiments. Like adsorbed normal serum, it depresses the surface potential and causes a shift in the isoelectric point; but there the similarity ends. The reagin-globulin is rendered water-insoluble by its firm combination with the lipid, exactly as any antibody is denatured upon combination with its specific antigen (bacteria, red cells, or dissolved protein). The hydrophobic films of reagin have five times as great an affinity for each other as the original lipid surfaces; accordingly, the critical potential is raised from its original value of 1 to 5 millivolts to 10 to 15 millivolts, that of particles of de-

natured globulin or of any antigen-antibody complex, and relatively small quantities of electrolytes (at serum pH, cations) suffice to depress the stabilizing potential below this critical level, with resultant aggregation and flocculation. In brief, a specific globulin combines with the colloidal particles of the antigen, conferring upon them the unstable properties of a suspension of denatured protein.

Like the antibody film on bacteria, or red cells, and unlike normal adsorbed protein, the reagin globulin on the lipid particle can adsorb ("fix") complement. When this protein film is destroyed by heat-coagulation, the complement-fixing property is lost; concomitantly, the specific groups of the lipid having been freed from the closely adherent reagin, the antigen becomes again active, able to react with more syphilitic serum.

These changes in the properties of reagin globulin upon its combination with the lipid antigen (denaturation) are in every sense analogous to those effected in any antibody by its specific antigen, and are probably due to the same, as yet unknown, factors. It has been suggested for bacterial and red cell "agglutinins" and protein "precipitins," that the groups of the antibody determining its specificity are also those which endow it with its hydrophilic properties; when these combine with antigen, residual free hydrophobic groups determine the surface properties of the complex. The same tentative hypothesis may be offered for the denaturation of reagin globulin by the lipid antigen.

The complete analogy between the flocculation reactions for syphilis and the so-called specific reactions (bacterial and red cell agglutination; protein precipitation) suggests that like agglutinins, precipitins, etc., reagin globulin represents an antibody response to products of infection.

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STUDIES IN THE SEROLOGY OF SYPHILIS

II. THE PHYSICAL BASIS OF THE WASSERMANN REACTION

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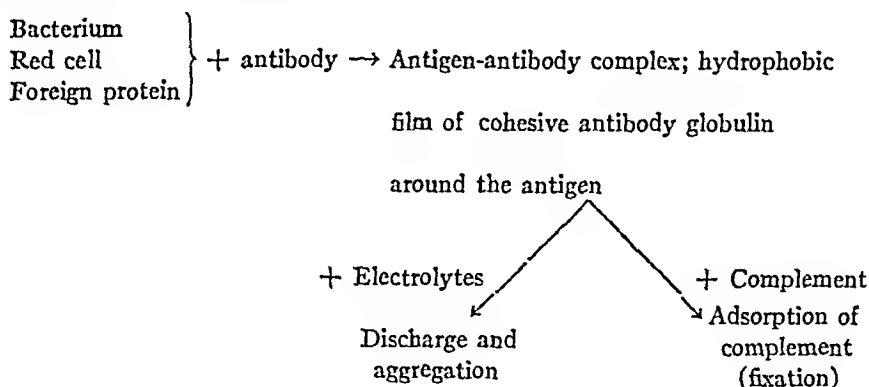
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A complete exposition of the theories which have been advanced to explain the Wassermann reaction would be beyond the scope of the present paper. The recent tendency is to regard it as a "colloidal phenomenon," which, in view of the fact that all the reagents are in colloidal solution, is a paraphrase of the problem rather than an explanation. Stern (1923, 1924) and Reiner (1929), finding that the addition of tannin to an antigen (organ-lipoid) suspension endows it with complement-fixing properties, have suggested that the Wassermann reaction is due to a similar dehydration of the antigenic particles by syphilitic serum. It is difficult to correlate such an hypothesis with the experimental fact that the complement-fixing properties of a biologically-sensitized antigen are completely destroyed at 100°C., while the antigen as such is thereby unaffected.

It has been shown (Northrop and de Kruif, 1922; Shibley, 1926; Mudd and Mudd, 1926, 1927; Eagle, 1929, 1930, 1) that the basic immune reaction involves the deposition of a film of denatured antibody globulin around the antigenic red cell, bacterium or foreign protein with which it has combined; and that both flocculation and complement fixation are secondary reactions. The former results from the discharge of this hydrophobic surface film of denatured antibody by electrolyte; the latter is an adsorption of complement by this same globulin film. The cause of the denaturation which endows the antibody globulin with these hydrophobic and complement-avid

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properties is as yet unknown; a tentative hypothesis was suggested which need not be discussed here.



The flocculation of lipid antigen by syphilitic serum is exactly analogous to these antigen-antibody reactions (Eagle, 1930, 3). Reagin globulin is denatured upon its combination with the lipid antigen, forming a hydrophobic sensitizing film with a critical potential of 10 to 15 millivolts, as contrasted with 1 to 3 millivolts for the original antigen. NaCl $N/15$ suffices to depress the surface charge below this critical level, allowing the cohesion of the sensitized particles, a cohesion primarily of the reagin globulin film, and only incidentally of the underlying lipid antigen.

It remains to show that, as in the case of the so-called specific reactions, the same globulin film which causes flocculation also determines complement fixation. At first sight, the high degree of correlation between the Wassermann and flocculation tests (Georgi, 1919; Mason, 1920; Sahlmann, 1922; Kritschewsky, 1928) would seem to establish our thesis. However, scattered evidence to the contrary in the literature (Wendlandt, 1920; Gloor and Klinger, 1920; Nathan, 1922), and the occasional discrepancies between the two types of test necessitate an empirical proof that precipitating and complement fixing reagin are identical.

1. a. If the Wassermann reagin is identical with the substance in syphilitic serum which is responsible for flocculation, it should be associated with the same fraction of serum protein, the globulin. The literature bearing on this point is quite conflicting.

Felke (1920-1921) thinks that the active substance is contained primarily in the albumen fraction; yet his own protocols show that removal of the globulin caused a certain proportion of positive sera to become negative. Gloor and Klinger (1920) state unqualifiedly that although the globulin fraction determines flocculation (Sachs-Georgi reaction), the albumen is the carrier of Wassermann reagin. On the other hand, Kapsenberg (1921) finds the globulin of a positive serum to be positive always, while the albumen fraction if positive, is weakly so. In a later paper (1924) he comes to the conclusion that this residual reagin in the albumen is due to an incomplete removal of globulin.

This source of error, which vitiates the results of Felke and of Gloor and Klinger, has been emphasized by Sahlmann (1922). Both the globulin precipitate and the supernatant fluid may be positive after the fractionation of syphilitic serum with CO_2 or HCl ; but since only a portion of the globulin (the so-called euglobulin) is thereby precipitated, no conclusion can be drawn as to the distribution of reagin between globulin and albumen. The experiments of Stern (1923, 1924) seem definitive. Such procedures as simple dialysis, or precipitation by CO_2 or HCl , cause an incomplete separation of globulin and therefore of reagin; but when all the globulin is precipitated, as by electrodialysis, all the Wassermann reagin is concentrated in the sediment, and the supernatant albumen is completely negative.

b. Another objection to the globulin theory of Wassermann reagin was raised by Skrop (1923). The active substance was stated to have a positive charge, migrating to the cathode in an electrical field, while globulin at serum pH ionizes as Na^+ globulinate⁻; the globulin ion being negatively charged and migrating to the anode. These experimental data have been vigorously discredited by Stern (1923). What was supposed to be reagin concentrated at the cathode was actually acid (pH 3.8 to 6.1) which accumulates during dialysis; the supposed complement-fixation it caused was a non-specific destruction. Furthermore, the fact that negatively charged particles adsorb reagin does not prove it to be positively charged: both negatively and positively charged substances may be adsorbed by negatively charged particles (*e.g.*, Kaolin).

Indeed, upon passing syphilitic serum through a series of 4 dry Berkefeld filters, we found that there had been a 30 per cent decrease of reagin-content, presumably due to adsorption by the negatively charged particles of the filters: but there was a parallel decrease in serum protein (25 per cent), proving that opposite charges are not a prerequisite for adsorption, and suggesting that serum protein is the carrier of the substance determining the reaction.

c. The constant association of the Wassermann reagin with serum globulin does not, of course, establish their identity. However, every effort to separate the two has failed. Forssman (1921) has stated that if the globulin precipitate obtained from syphilitic serum by acidification with acetic acid is redissolved in NaCl, and the solution shaken with ether, it becomes Wassermann negative; but that if the ether extract is allowed to evaporate onto this solution, it again becomes Wassermann positive. Apparently, this proves that reagin is an ether-soluble lipid merely carried down with the globulin. However, his whole case is given away by the fact that not only the extract, but even normal ether can restore the positivity of the extracted globulin solution.

Upon repeating his experiments it is found that when a solution of serum globulin is shaken with ether, most of the protein is coagulated, probably due to adsorption and denaturation at the ether-water interfaces in the emulsion. The disappearance of the positive reaction is therefore due to an irreversible coagulation of the reagin-globulin, and not to an extraction of a hypothetical reagin-lipoid. When ether is layered onto this suspension of denatured globulin and allowed to evaporate, one obtains a highly anticomplementary solution, due to acids contained in the ether. The supposedly restored positive Wassermann is in reality a non-specific destruction of complement by acid impurities in the ether.

To summarize, Wassermann reagin is constantly associated with the serum globulin, confirming its identity with the substance which causes the flocculation of antigen. Further evidence is given in the following sections.

2. In every positive Wassermann reaction there is microscopically visible aggregation of the lipid particles into small aggregates of two or three particles (Jacobsthal, 1911). There are no large visible or sedimenting clumps solely because the antigen is used in too small quantities to give optically visible aggregates.

3. It is well known (Jacobsthal, 1911; Ravenel and Dulaney, 1925; Wassermann, 1921) that the antigen lipid suspension alone does not fix complement, while the lipid-protein aggregates obtained by adding this suspension to syphilitic serum fix complement powerfully.

4. It is also known that heat, which would coagulate the film of globulin, destroys this acquired complement-fixing property (Stern,

1929). Simultaneously, the antigen particles regain their ability to fix complement with syphilitic serum, a property previously masked by the surface film of denatured reagin with which they had already combined (Paper I of this series; Table VI).

5. The fact that certain conditions will inhibit complement fixation without affecting flocculation, or *vice versa*, does not disprove the identity of the substance causing the two. The basic reaction in any serological test for syphilis is always the same: particles of lipoid antigen combine with reagin globulin, which is deposited as a film of denatured protein around the underlying lipoid. The subsequent aggregation of these sensitized particles due to the hydrophobic properties of this protein film and the subsequent adsorption due to its avidity for complement midpiece are entirely different physico-chemical processes, with different optimal conditions. Complement fixation takes place at pH 5.8 to 8.2, and at a NaCl concentration of 0.07 to 0.25 N, with inhibition on either side of this optimal range; it is only slightly affected by volume and shaking, is completely suppressed by relatively slight concentrations of bivalent cations, and because of the thermolability of complement, must be carried out at $<40^{\circ}\text{C}$. (Eagle, 1929, 3). The cohesion of the protein-coated particles on the other hand takes place most rapidly at $\text{pH } 3.5 \pm 0.5$, is enormously accelerated by shaking, by decrease in total volume, by bivalent cations or by an increase in temperature up to 56°C .

One can therefore pick any number of variables which would have opposite effects upon the two types of reaction: but obviously this is due to differences in the types of reaction and does not connote a different causal factor.

6. Contrary to the statement of Gloor and Klinger (1920), complement-fixing reagin is completely removed by the antigen.

If an excess of antigen is added to syphilitic serum, and the precipitate removed by centrifugation (or by Berkefeld filtration) it is found that the supernatant fluid no longer gives a positive flocculation test upon a second addition of antigen; moreover, it no longer fixes complement, *i.e.*, it gives a negative Wassermann test. Provided only that sufficient antigen is used to combine with all the reagin, more than 99 per cent of the complement-fixing substance is concentrated in the precipitate (Table I, Protocol 1).

Protocol 1

To x cc. of strongly positive syphilitic serum were added $1/6$ x cc. of an alcoholic solution of cholesterinized antigen and NaCl N/7 to 2 x cc. The precipitate was removed by a) prolonged centrifugation and b) Berkefeld filtration, and the complement fixing properties of the original serum, precipitate and supernatant fluid determined by a method described elsewhere (Eagle, 1930, 2). The unit of Wassermann reagin is taken as that necessary to give 75 per cent complement fixation: the titre is expressed as the dilution of serum containing 1 unit.

TABLE I

	0.4 cc.	0.2 cc.	0.1 cc.	0.05 cc.	0.025 cc.	Units reagin per cc.	Flocculation test
Serum 1:2, per cent fixation (x cc. + 0.4 cc. complement 1:10 + 0.4 cc. antigen).....	>90	>90	>90	80	50	22	++++
Supernatant, after centrifugation, per cent fixation (x cc. + complement 1:10; anticomplementary determination).....	50	25	10	0	0	2 units anticomplementary	0
Supernatant after centrifugation, per cent fixation (x cc. + complement + antigen; anticomplementary + reagin).....	50	25	10	0	0	2 units anticomplementary + reagin	0
Supernatant after centrifugation; per cent fixation by free reagin.....	<10	0	0	0	0	0 reagin units	0
Berkefeld filtrate per cent fixation x cc. + complement + antigen.....	(20) (?)	0	0	0	0	0	0

As seen in Table I, the supernatant fluid after centrifugation contains less than $1/20$ as much free reagin as the original serum. It is somewhat anticomplementary, due to the fact that small clumps of antigen sensitized with reagin are not completely carried down; but a further addition of antigen does not increase the degree of complement fixation, *i.e.*, there is no free reagin. Berkefeld filtration removes these

sensitized antigen particles completely, with the result that the clear filtrate is no longer anticomplementary, and contains no demonstrable reagin.

SUMMARY AND DISCUSSION

The substance in syphilitic serum which is responsible for the Wassermann reaction, like that which determines the diagnostic flocculation tests, is associated with the globulin fraction of serum.

Every positive Wassermann is accompanied by microscopic (or submicroscopic) aggregation, which is not an essential feature of the reaction; conversely, after every positive flocculation test, the washed precipitate will fix complement. An excess of antigen removes both flocculating and complement-fixing substances completely (>95 per cent). Heating the lipoid-reagin precipitate to 100° for 1 minute destroys the sensitizing film of reagin globulin; the avidity for complement disappears simultaneously.

Both the flocculating and complement-fixing properties of syphilitic serum are therefore determined by the same substance, a specifically altered fraction of the serum globulin, reagin. The Wassermann reaction is thus entirely analogous to complement fixation by any antigen-antibody complex. The same film of denatured serum globulin which sensitizes the antigen particles, whether red cells, bacteria, protein, or colloidal lipid particles, to discharge and aggregation by electrolytes, also endows them with an avidity for complement.

The pathogenesis of reagin will be discussed in a forthcoming paper.

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STUDIES IN THE SEROLOGY OF SYPHILIS

III. EXPLANATION OF THE FORTIFYING EFFECT OF CHOLESTERIN UPON THE ANTIGEN AS USED IN THE WASSERMANN AND FLOCCULATION TESTS

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(Received for publication, July 15, 1930)

Soon after the discovery that an alcoholic extract of normal tissue could be used in the serum diagnosis of syphilis (Landsteiner, Muller and Pötzl, 1907), it was found by Browning, Cruikshank and Mackenzie (1910) that small quantities of cholesterin added to such an extract increased its sensitivity markedly. Although its use both in the complement fixation and flocculation tests (Kahn, Sachs-Georgi, Meinicke, Hinton, etc.) has since become almost universal, there is as yet no satisfactory explanation of its effect.

It is certainly not in itself antigenic (Protocol 1 and Table I): 100 times as much cholesterin as the fixing quantity of antigen has no effect with syphilitic serum. As Maltaner (1930) recently showed, its activity is not to be ascribed to traces of activated ergosterol: weight for weight, the latter is no more effective than pure cholesterol. Most of the text-books speak of an obscure physico-chemical effect. The explanation of its action is the subject of the present paper.

Protocol 1

1 cc. of an alcoholic extract of beef heart containing $1\frac{1}{2}$ per cent total solid was dropped with shaking into 40 cc. NaCl ≈ 7 . To decreasing quantities of this colloidal lipoid suspension were added 0.4 cc. of 1:10 guinea pig complement, 0.4 cc. of 1:4 strongly positive syphilitic serum and NaCl ≈ 7 to 1.2 cc. After 4 hours at ice box temperature and $\frac{1}{2}$ hour at 37°C ., 0.8 cc. of strongly sensitized sheep cells were added to each tube, and the time for complete

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hemolysis determined in seconds. The degree of complement fixation was calculated from hemolysis time by a method already described (Eagle, 1929, 1; 1930, 1).

An exactly similar experiment was carried through at the same time with the same reagents, using 1½ per cent cholesterin instead of antigen, diluted with water instead of saline. An individual experiment is summarized in Table I.

I. Cause of the Stable Dispersion of Cholesterin in the Presence of Antigen

Both the antigen and cholesterin are water-insoluble and alcohol-soluble, but they differ markedly in their affinity for water. The anti-

TABLE I
Cholesterin as Such Has No Effect with Syphilitic Serum

cc. of 1:10 dilution of 1½ per cent antigen (or cholesterin).....	0.4	0.2	0.1	0.05	0.025	0.0125	0.006	0.003
Per cent fixation by x cc. antigen + 0.1 cc. syphilitic serum.....	>90	>90	>90	>90	>90	90	50	30
Anticomplementary control of antigen, per cent destruction.....	25	10	0	0	0	0	0	0
Actual fixation by antigen.....	>90	>90	>90	>90	>90	90	50	30
Apparent fixation by x cc. cholesterin + 0.1 cc. syphilitic serum, per cent.....	25	10	0	0	0	0	0	0
Anticomplementary control cholesterin, per cent destruction.....	25	10	0	0	0	0	0	0
Actual fixation by cholesterin, per cent.....	0	0	0	0	0	0	0	0

gen, although insoluble as such, can be readily peptized by dropping the alcoholic solution into an excess of water. The colloidal solution obtained is not only stable, but is remarkably hydrophilic for a water-insoluble substance, remaining colloidally dispersed until the surface potential of the constituent particles has been reduced to 1 to 5 millivolts (0.75 to 1.5 N NaCl), when they cohere to form unstable aggregates. Although the suspension is optically homogeneous, these discrete particles can be seen by dark field illumination as minute refractile bodies in active Brownian motion.

An alcoholic solution of cholesterin on the other hand cannot be so peptized. Dropped into water, needle crystals of cholesterin form immediately, which despite their very high mutually repellent surface

charge, quickly coalesce to form unstable aggregates. The difference in affinity to water can be expressed as the ratio of their critical potentials:

$$\frac{> 100 \text{ (cholesterin)}}{1-5 \text{ (antigen)}}$$

However, when the cholesterin is added to an alcoholic solution of antigen and the mixture is dropped into an excess of water, instead of obtaining a precipitate of cholesterin and a colloiddally dispersed antigen, both remain colloiddally dispersed in an optically homogeneous solution. Corresponding to its greater opalescence, there are more minute particles to be seen, of somewhat larger size than those formed by antigen alone, but no cholesterin as such. Obviously, each particle contains antigen and cholesterin, and the complex is more efficient in both complement fixation and precipitation than the antigen particle alone.

To hypothecate a chemical combination as the explanation of the peptization of the cholesterin would be begging the question. When one drops an alcoholic solution of cholesterin into an aqueous colloidal suspension of antigen, it precipitates just as it would in pure water. There is certainly no reaction between the two substances in alcoholic solution, and they can be readily dissociated from their complex colloidal sol. Nor would such an hypothesis explain the greater sensitivity of the lipoid-cholesterin sol in the diagnostic reactions unless we introduce as another assumption that the hypothetical compound is more reactive than the lecithin antigen alone. We find an analogy in the detergent action of soap upon fatty acids, silica, etc. The soap is adsorbed by the particles of dirt, forming a hydrophilic film which allows them to pass into the solution, and prevents their cohesion (Freundlich, 1922). Similarly, we can hypothecate that when the alcoholic antigen-cholesterin solution is dropped into water, the natural tendency of the cholesterin molecules to cohere is prevented by the fact that, as submicroscopic aggregates form, they adsorb the lecithin antigen; this would form a protective film, preventing any further aggregation and allowing the particles to remain colloiddally dispersed. Each particle seen microscopically would thus contain both antigen and cholesterin, not in any haphazard distribution, but in a definite

arrangement: a nucleus of cholesterin surrounded by an adsorbed protective antigen surface of indeterminate thickness. (See Fig. 2.)

The forces determining this adsorption would be those of interfacial tension. If

$$\rho \text{ cholesterin-water} > \rho \text{ cholesterin-antigen} + \rho \text{ antigen-water,}$$

that is, if the surface tension of cholesterin against water were greater than the sum of the antigen-water and cholesterin-antigen interfacial tensions, then the antigen would be adsorbed, since the total free surface energy would be thereby diminished. Unfortunately, there are no methods for measuring solid-solid or solid-liquid interfacial tensions. Qualitatively, the complete insolubility of cholesterin, as contrasted with the ease of peptizing antigen, as well as the known low surface tension of lecithin against air (Price and Lewis, 1929) suggest that $\rho \text{ Chol.}-\text{H}_2\text{O} > \rho \text{ Ant.}-\text{H}_2\text{O}$, but we have no indication of the magnitude of $\rho \text{ Ant.}-\text{Chol.}$

There are, however, several other methods of verifying the suggested explanation. If surface-active antigen actually causes the stable dispersion of cholesterin by forming a protective film around minute particles of cholesterin, preventing their further aggregation, then (1) the surface properties of the individual antigen-cholesterin particles should be those of the antigen, the properties of cholesterin being completely masked by the superimposed film; (2) any substance with the same physical properties as antigen should cause a similar dispersion of cholesterin in water; (3) any substance of the same physical properties as cholesterin should be as readily peptized by the lipoid antigen. The empirical data follow.

A. Surface Properties of the Antigen-Cholesterin Particles.—

1. *Isoelectric Point.*—The isoelectric point of the lecithin antigen is pH 1.9 (Eagle, 1930, 2), corresponding to the fact that it contains both phosphoric acid and fatty acid radicals, and a comparatively weak organic base. Although that of cholesterol is usually given as pH 3–4 \pm , it is difficult to understand from its chemical structure why it should be amphoteric, containing as it does only one active group, an alcohol. Accordingly, it was not surprising to find that of six different samples, no two had the same cataphoretic isoelectric point

(Table II). Presumably, therefore, the so-called isoelectric point of cholesterin is due to an associated impurity (glycocholl, etc.).*

The point of interest in the present connection, however, is that no matter what the original isoelectric point of the particular sample of cholesterin, that of the particles of an antigen-cholesterin sol is always the same, pH 1.9 ± 0.2 , the isoelectric point of antigen alone; indicating that the surface of the particles is not a mosaic of cholesterin and antigen, as it would be in a haphazard distribution, but antigen only, surrounding a core of cholesterin. (Table II.)

TABLE II

Showing that the isoelectric point of the particles of an antigen-cholesterin sol is independent of that of the cholesterin, depending solely upon the antigen surface.

Manufacturer	Isoelectric point of cholesterin particles	Isoelectric point of particles of antigen-chol. sol (ant.:chol. ratio = 1/1)
Hollister.....	3.3	1.8
Pfanstiehl.....	2.1	1.9
Eimer and Amend.....	3.2	1.95
Merck.....	3.4	2.0
Laboratory Spec. 1, from gallstones.....	2.5	1.8
“ “ 2, “ “	3.6	1.8

2. *Critical Potential and Coagulation Value.*—An alcoholic solution of antigen alone when dropped into water forms a very stable suspension at any pH > 1.9. Thus, at pH 7.4 the critical potential, the minimum compatible with stability, is 1 to 3 millivolts, a very low value for a water-insoluble substance. Its flocculation value, *i.e.*, the amount of electrolyte necessary to reduce the surface potential below this critical value, is 0.5 to 1.5 N for univalent cations. The rather wide limits given are due to the fact that the stability of the sol varies with its concentration (Eagle, 1930, 2).

Cholesterin on the other hand, despite its very high cataphoretic potential (>100 millivolts) flocculates even in water. If the particles

* The extremely low buffer value of an alcoholic solution of cholesterin as contrasted with one of truly amphoteric antigen, is further proof that its isoelectric point is due to an impurity.

of the antigen-cholesterin sol consisted of the two substances in a haphazard distribution, the surface would consist of a mosaic of antigen and cholesterin, and its surface properties would be intermediate between the two. Instead, even with 2 parts of cholesterin to one of antigen, the critical potential of the complex antigen-cholesterin sol is still 1 to 5 millivolts, the value of pure antigen, instead of about 70, as it would be if the particles contained antigen and cholesterin in a statistical distribution (Protocol 2 and Table III).

Protocol 2

Alcoholic solutions of antigen (solid content 1.6 per cent) containing increasing quantities of cholesterin were shaken with an equal volume of NaCl *N*/7. The suspensions were centrifuged and made up in H₂O to their original volume, forming suspensions containing 0.8 per cent antigen lipoid plus varying quantities of added cholesterin. 0.2 cc. of each suspension were added to 3.7 cc. of NaCl of varying concentration, and brought to approximately pH 7.4 with 0.1 cc. of phosphate buffer *N*/15. The figures in the body of Table III represent degrees of flocculation after 24 hours at room temperature. The slight decrease in stability with added cholesterin is due entirely to the increased number of particles, those most readily flocculated.

The only explanation for this anomalous finding is that cholesterin is concentrated in the center of the particles as its core, antigen forming a surface film which determines the surface properties of the complex particle.

TABLE III

Showing that cholesterin does not appreciably affect the coagulation value or critical potential of the antigen sol (pH 7.4).

Composition of alcoholic solution	Cholesterin ÷ antigen	Flocculation after 24 hours						Isoelectric point	Coagulation value (NaCl)	Critical potential, millivolts
		<i>N</i> /1	<i>N</i> /2	<i>N</i> /4	<i>N</i> /8	<i>N</i> /16	<i>N</i> /32			
Antigen lipoids 1.6%.....	0	0	0	0	0	0	0	1.9	<i>N</i> /1	<2
Antigen lipoids 0.8% + cholesterin 0.4%.....	1/2	±	0	0	0	0	0	1.9	<i>N</i> /1	<2
Antigen lipoids 0.4% + cholesterin 0.4%.....	1/1	4 ±	0	0	0	0	0	2.0	<i>N</i> /2— <i>N</i> /1	<2
Antigen lipoids 0.2% + cholesterin 0.4%.....	2/1	4	2	0	0	0	0	1.7	<i>N</i> /2— <i>N</i> /1	2-3
Antigen lipoids 0 + cholesterin 0.4%...		4	4	4	3	3	3	3.6	0	>100

B. Peptization of Other Water-Insoluble Substances by Antigen.—

The explanation suggested for the formation of the stable lipid-cholesterin sol implied as a second and necessary corollary that any substance of the same physical properties as cholesterin should be similarly peptized by antigen. The most diverse types of substances were tested, all water-insoluble, alcohol soluble and all flocculating either immediately or within a few hours when the alcoholic solution is dropped into an excess of NaCl $\frac{N}{7}$. In the majority of cases the presence of antigen in the alcoholic solution allowed the substance to

TABLE IV

Effect of Antigen upon the Stability of a Series of Alcohol-Soluble, Water-Insoluble Substances

Substance	Alcoholic solution dropped into NaCl $\frac{N}{7}$	Alcoholic solution + antigen dropped into NaCl $\frac{N}{7}$
Phenyl salicylate.....	Precipitate	Stable opalescent sol
Gums: Mastic, elemi, copal, sandarac, benzoin, etc.*.....	"	"
Palmitic acid.....	"	Precipitate
Benzophenone.....	"	Stable opalescent sol
Ethyl stearate.....	"	"
α -Naphthonitrile.....	"	"
Triphenylphosphine.....	"	"
Cetyl alcohol.....	"	Precipitate
<i>p</i> -Nitrobromobenzene.....	"	Stable opalescent sol
Benzil.....	"	"
etc., etc.		

* The precipitate obtained may be a resinous impurity.

be colloiddally dispersed irrespective of its chemical nature, corroborating the hypothesis that the hydrophilic antigen forms a protective film around the hydrophobic particles in *statu nascendi*, preventing their further aggregation into sedimenting clumps (Table IV).

For all these substances, then,

$$\rho \text{ substance-water} > \rho \text{ substance-antigen} + \rho \text{ antigen-water};$$

as in the case of cholesterin, their interfacial tension against water exceeds that of the two surfaces formed by the adsorption of the lecithin

antigen. When the alcoholic solution (of antigen + x) is dropped into water, minute particles of x therefore adsorb antigen while they are still $<1\mu$ in diameter. The complex particles formed have a surface of hydrophilic antigen, and are as stable as antigen particles of the same size.

C. Peptization of Cholesterin by Substances Other Than "Antigen."—

Conversely, not only the alcoholic extract of beef heart, but any similar substance will cause the colloidal dispersion of cholesterin, again indicating that the phenomenon is determined, not by a specific chemical affinity between antigen and cholesterin, but by their physical properties. Thus, if cholesterin is added to an alcoholic extract of dried milk (or of egg yolk) and the mixture dropped into an excess of water or saline, one obtains a stable milk (egg) lipid-cholesterin sol. Similarly, an alcoholic extract of any animal tissue will cause a stable colloidal dispersion of aggregates of cholesterin before they become sufficiently large to sediment. Indeed, an aqueous solution of any substance with a low interfacial tension against both cholesterin and water should have the same effect. To test this, an alcoholic cholesterin solution was dropped into solutions of serum, Na-oleate, Na glycocholate, and Na taurocholate in NaCl $N/7$, all surface-active substances of varying efficiency. In all four, there was obtained an opalescent, but stable colloidal suspension of cholesterin, contrasting with the coarse rapidly sedimenting aggregates obtained in saline alone. Here again we may write that

$$\rho \text{ cholesterin-water} > \rho \text{ cholesterin-serum} + \rho \text{ serum-water} \\ \text{[or]} > \rho \text{ cholest.—Na-oleate, etc.} + \rho \text{ Na-oleate-water, etc.}$$

To summarize, since the particles of an antigen-cholesterin sol have the same isoelectric point, critical potential and coagulation value as antigen alone; since any similarly surface active substance (egg or milk-lipoid, etc.) can be substituted for the beef heart lipid antigen as the peptizing agent; and since antigen will cause a similar colloidal dispersion of any hydrophobic alcohol-soluble substance like cholesterin: we conclude that antigen causes the colloidal dispersion of cholesterin by forming a protective film around minute aggregates of cholesterin before they exceed the limits of colloidal stability, endowing them with its own stable surface properties, and preventing their further aggregation into sedimenting clumps.

The phenomenon is therefore analogous to the detergent action of soap. The adsorption of a surface-active hydrophilic substance (antigen, soap) by minute particles of a hydrophobic substance (cholesterin, dirt) allows the latter to form a stable colloidal suspension in water. The difference between the two is that the initially large particles of dirt are peptized by friction, and adsorb the soap as they break off; while the cholesterin, beginning as a molecularly dispersed alcoholic solution, would form very large unstable aggregates in water were it not for the fact that the tiny particles adsorb antigen before they become sufficiently large to sediment.

II. Physical Properties of the Antigen-Cholesterin Sol, as Contrasted with Antigen Alone

As already shown, the surface properties of the individual particles in the complex sol are determined by the adsorbed antigen surface; their isoelectric point and critical potential are therefore approximately the same as those of particles of pure antigen. There is, however, a very great difference in the degree of dispersion.

In any sol, the size of the particles varies widely. When an alcoholic solution of antigen alone is dropped into an excess of saline, it forms a highly dispersed colloidal solution in which only a few of the particles are within the limits of dark field visibility, probably less than 1/10. The remainder are even smaller, probably $<0.05\mu$ in average diameter. Corresponding to the small average size of the particles, the turbidity of the suspension is very slight. However, the addition of relatively small quantities of cholesterin to the antigen causes an enormous increase in the number of particles visible by dark field illumination. Thus, 1 part of cholesterin to 3 of antigen increases the number of visible particles twenty-fold, sixty times more than a similar quantity of antigen. Not only the number, but the average size of these visible particles increases *pari passu* with the amount of added cholesterin, as does the turbidity (Fig. 1). Clearly, when the cholesterinized antigen is dropped into saline, the cholesterin aggregates very rapidly; before an adequate protective cover of antigen can be adsorbed, most of the particles exceed in size the largest of these formed by antigen alone. And since the actual mass of the cholesterin is very small, these larger antigen-cholesterin particles must be obtained at the ex-

pense of the very small submicroscopic particles formed in the absence of cholesterol (Fig. 2). The net result is therefore a coarsened dispersion state of antigen, intermediate between the finely dispersed sol formed by antigen alone and the large visible clumps formed by cholesterol.

As the proportion of cholesterol to antigen in the alcoholic solution

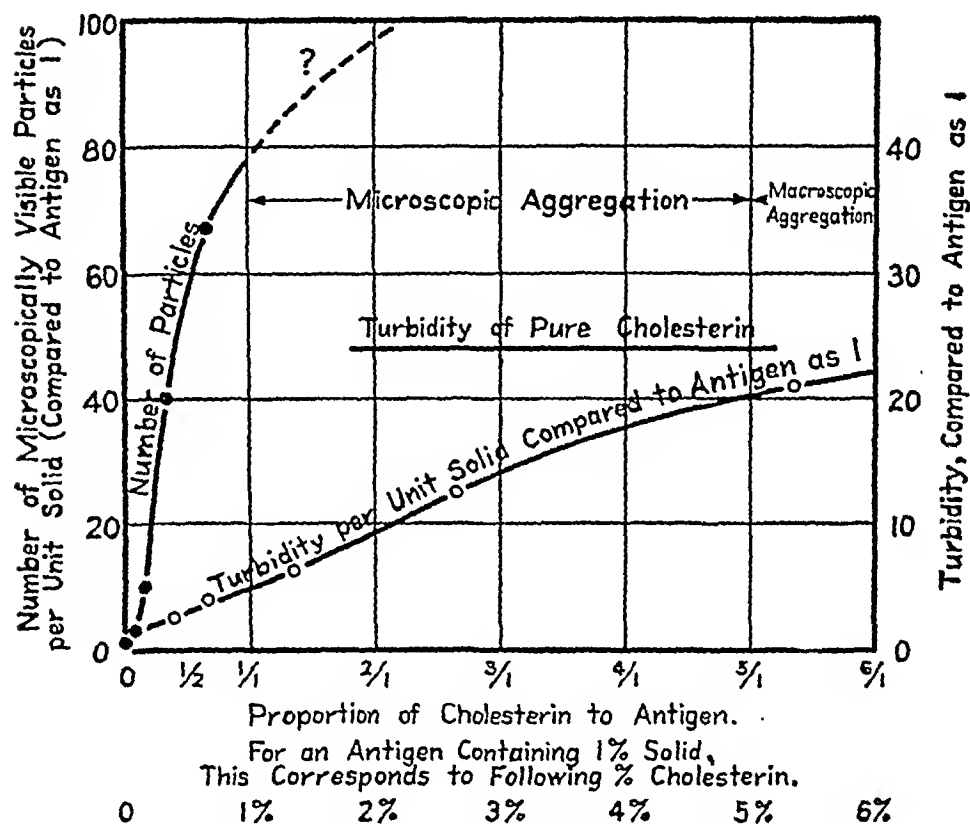


FIG. 1. Effect of cholesterol upon physical properties of the antigen sol (see Table V).

is increased (Table V), the microscopically visible particles in the aqueous sol become more and more numerous, and of larger average size; but they remain discrete until the cholesterol-antigen ratio is about 1/1. Any further increase in the proportion of cholesterol results in the formation of tiny microscopic aggregates; however, these do not become sufficiently large to sediment or be visible microscopically

until the alcoholic solution contains 5 to 7 parts of cholesterol to 1 of antigen. At this critical ratio, the sol is no longer homogeneous and opalescent, but becomes cloudy. With even more cholesterol, there is progressively more rapid flocculation and sedimentation of coarse grossly visible aggregates.

Paralleling the coarsened dispersion state of the antigen caused by cholesterol, the turbidity of the sol per unit solid increases, approaching as its maximum value that of a suspension of pure cholesterol. It should be noted that as little as 1 part of antigen to 8 of cholesterol causes a significant change in the turbidity, *i.e.*, of its degree of dis-

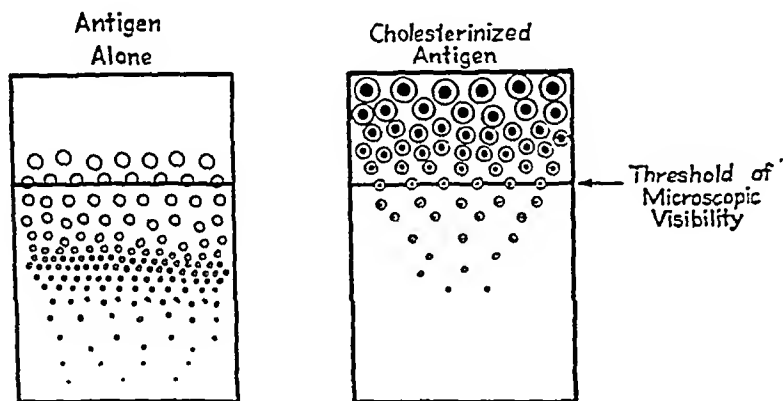


FIG. 2. Diagrammatic representation of effect of cholesterol upon particle number and size in an antigen sol (solid black core indicates cholesterol).

persion, in keeping with the fact that it forms a thin surface film. These data are all summarized in Protocol 3 and Table V.

Protocol 3

A series of tubes with varying proportions of antigen and cholesterol were set up as outlined in Table V, and dilutions made by dropping into saline. The number of particles were counted in the Petroff-Hausser counting chamber (1/50 mm. deep) by dark field illumination. The Brownian motion of the particles made counting difficult, and the figures given are necessarily approximate.

Turbidity measurements were made roughly by determining the degree to which each suspension had to be diluted in order to approximate the turbidity of the pure antigen sol. The results are summarized in Table V and Figs. 1 and 2.

III. Explanation of the Effect of Cholesterin in the Wassermann Reaction

Complement fixation in the Wassermann reaction is an adsorption of complement midpiece by the same film of denatured reagin-globulin which sensitizes the particle to flocculation by electrolyte (Eagle, 1930, 3). The degree of fixation increases with the amount of antigen used (Table I), and since the reaction takes place at the surface of the particles, this is the equivalent of saying that it increases with the total surface area.

A priori, this would imply that the more finely divided a given quantity of antigen, the more efficient is the resulting sol in complement fixation: for surface increases with progressive subdivision. Actually, however, as the following experiment shows, within the range covered by the particles of antigen and antigen-cholesterin sols, a coarsely dispersed antigen dilution is much more sensitive in complement fixation, despite the fact that it has less surface, confirming the findings of Kermack and Spragg (1929).

If an alcoholic solution of antigen is dropped into an excess of NaCl $\text{N}/7$, it forms a highly disperse, faintly opalescent sol in which but few particles are visible by dark field examination. But if the saline is dropped into the antigen, keeping the quantities the same, a much more turbid suspension is obtained (Sachs and Rondoni, 1909) containing about 20 times as many visible particles, and correspondingly fewer small ones. The total surface area of this coarse water-into-antigen sol is much less, yet it is about twice as efficient in complement fixation with syphilitic serum (Protocol 4 and Table VI). Per unit surface, the larger particles are therefore at least 5 times as efficient.

Protocol 4

1 cc. of an antigen containing $1\frac{1}{2}$ per cent solid matter was dropped into 39 cc. NaCl $\text{N}/7$. The transparent, very slightly opalescent sol contained 1 to 2 particles per 1/20,000 sq. mm. (2 to 4×10^4 per cubic centimeter) as counted by dark field illumination in the Zinsser-Hauser apparatus. When 39 cc. of NaCl $\text{N}/7$ were added with shaking to 1 cc. of the same antigen a markedly opalescent sol was obtained containing 4 to 6×10^5 visible particles per cubic centimeter, an increase of 20 fold. To x cc. of each antigen were added 0.4 cc. of guinea pig complement 1:10, 0.1 cc. of a strongly positive syphilitic serum and NaCl $\text{N}/7$ to 1.2 cc. The degree of fixation after 4 hours at ice box and $\frac{1}{2}$ hour at 37°C . was determined by a method already described (Eagle, 1930, 1). The results are summarized in Table VI.

The fact that cholesterin causes just such a coarse dispersion of the antigen when the mixture is dropped into saline, as compared with the finely dispersed sol formed by antigen alone, completely explains the greater efficiency of the cholesterinized antigen in the Wassermann reaction.

The basic cause of this greater efficiency of large particles is of only theoretical importance. There are two possibilities: 1) either the larger antigen particles have a much greater avidity for reagin, or 2) having combined with antigen, the film of denatured reagin is a more efficient adsorbent for complement when it is spread around large particles. As shown by the following experiment, the first is the correct explanation.

TABLE VI

Contrast in the Complement-Fixing Efficiency of Antigen-into-Water and Water-into-Antigen Sols of the Same Solid Content

Antigen cc. of 1:40 dilution.....	0.4	0.2	0.1	0.05	0.025
Per cent complement fixed by x cc. water-antigen sol + 0.1 cc. of same positive serum.....	>90 (4+)	>90 (4+)	80 (4+)	45 (0)	25 (0)
Per cent complement fixed by x cc. antigen-water sol + 0.1 cc. positive serum.....	80 (4+)	70 (3+)	40 (0)	20 (0)	10 (0)

Decreasing quantities of a strongly positive serum were incubated for 1 hour with 0.4 cc. of a) 1:40 antigen, b) 1:40 cholesterinized antigen. At the end of this time, an additional 0.1 cc. of 1:40 cholesterinized antigen was added to all the tubes. After 15 minutes, 0.4 cc. of complement was added to each tube, and the degree of fixation determined after $\frac{1}{2}$ hour at 37° by a method described elsewhere (Eagle, 1930, 1).

The combination between antigen and reagin is an irreversible one. The second dose of antigen could therefore combine only with free reagin not previously bound. As seen in Table VII, 75 per cent of all the reagin remains free after 2 hours' incubation with cholesterin-free antigen: the subsequent addition of as little as 0.1 cc. binds almost all this free reagin within 15 minutes, as shown by the degree of com-

plement fixation. Cholesterinized antigen, on the other hand, leaves almost no reagin free: accordingly, subsequently added antigen has practically no effect upon the degree of complement fixation.

TABLE VII
Cholesterinized Antigen Has Greater Avidity for Reagin

1:8 Syphilitic serum, cc.....	0.4	0.2	0.1	0.05	0.025	Interpretation
a) 0.4 cc. antigen (no cholesterin) 75 minutes; per cent fixation.....	50	25	—	—	—	Weak fixation by cholesterin free antigen
b) As in a); 0.1 cc. cholesterinized antigen added after 60 minutes; per cent fixation.....	>90	70	40	—	—	Reagin left free by non-cholesterinized antigen bound by supplementary cholesterinized antigen
a) 0.2 cc. cholesterinized antigen 75 mins.; per cent fixation.....	>90	80	50	25		Strong fixation by cholesterinized antigen
b) As in a); 0.4 cc. cholesterinized antigen added after 60 mins.; per cent fixation.....	>90	80	50	25		No reagin left free by cholesterinized antigen

TABLE VIII

Alcoholic extract of beef heart (antigen—2 per cent solid content), cc.....	1.6	1.6	1.6	1.6	0.8	0.8	0.4	0.2
Cholesterin (2 per cent solution in alcohol), cc..	0	0.1	0.2	0.4	0.4	0.8	0.8	0.8
Alcohol, cc.....	0.4	0.3	0.2	0	0.8	0.4	0.8	1.0
Cholesterin: antigen ratio.....	0	1/16	1/8	1/4	1/2	1/1	2/1	4/1
Dilution in NaCl N/7. 1 cc. of alcoholic mixture into x cc. total volume; dilutions obtained contain same quantity of antigen....	1-80	1-80	1-80	1-80	1-40	1-40	1-20	1-10

In brief, the effect of cholesterin in increasing the sensitiveness of the antigen in the Wassermann reaction is due to the fact that the larger particles formed by cholesterinized antigen when it is diluted

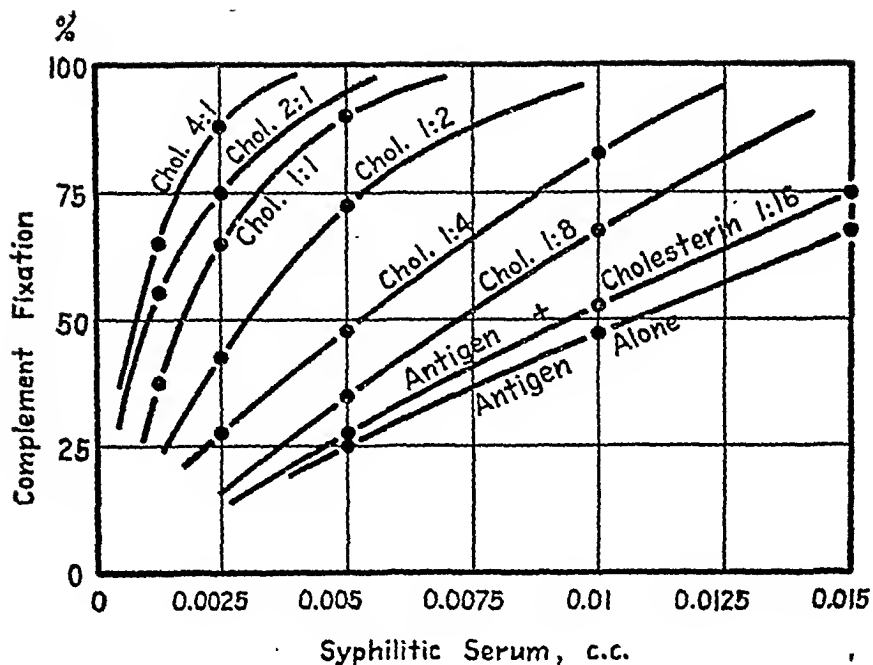


FIG. 3. Effect of cholesterol upon efficiency of antigen in the Wassermann reaction.

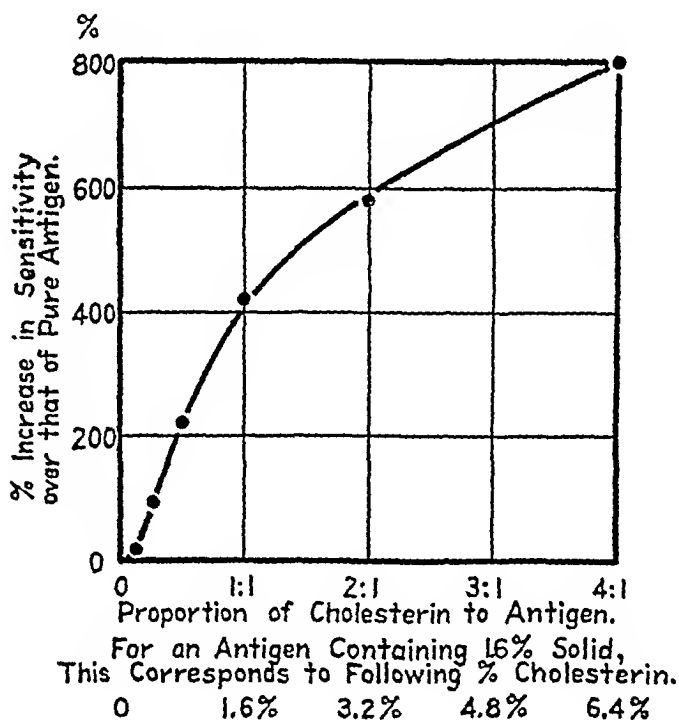


FIG. 4. Effect of cholesterol upon efficiency of antigen in the Wassermann reaction.

with water or saline have a markedly greater avidity for reagin than the submicroscopic lipid particles formed by antigen alone.

A very significant verification of practical importance is found in the following experiment. The amount of cholesterol used to sensitize the antigen for the Wassermann reaction varies anywhere from 0.05 to 0.2 per cent. The per cent of solids in the antigen is also variable, ranging from 1 to 2 per cent, a maximum cholesterol antigen ratio of 1/5 and a minimum of 1/100. As already shown, the effect of cholesterol upon the particle size in an antigen sol does not stop at this ratio, but continues indefinitely, the only limiting factor being its solubility in alcohol.

If our thesis is correct, *i.e.*, if the sensitizing effect of cholesterol upon Wassermann antigen is due solely to its effect upon the particle size, this effect should not stop at the commonly employed cholesterol: antigen ratio of 1/5, but should increase indefinitely so long as there is any observable effect upon particle number and size (Protocol 5 and Figs. 3 and 4).

Protocol 5

A series of antigen dilutions in NaCl $\times 7$ were prepared as outlined in Table VIII, each containing the same amount of antigen, but increasing quantities of cholesterol.

The minimum quantity of a strongly positive syphilitic serum which would give 75 per cent complement fixation (a 4+ reaction) was determined with 0.4 cc. of each antigen dilution and 0.4 cc. of 1:10 complement by a method described in detail elsewhere (Eagle, 1930, 1). The experimental data are summarized in Figs. 3 and 4.

As predicted, the fortifying action of cholesterol continues indefinitely. An increase is still effective when the cholesterol antigen ratio is already 2/1, *i.e.*, some 10 times as much as is used routinely. For an antigen containing 1.5 per cent solid, this means that the addition of cholesterol up to 3 per cent does not exhaust the possibilities of sensitization. The obvious implication is that cholesterol should be added up to the limit of its alcohol solubility: antigen containing 0.5 per cent cholesterol is roughly $1\frac{1}{2}$ times as sensitive as one containing but 0.2 per cent. Even more suggestive is the fact that the discovery of a very alcohol-soluble substance similar to cholesterol would increase

the sensitivity of the Wassermann antigen at least two-fold, for it could be added up to 1, 2 or even 3 per cent concentration (see Section V).

IV. Explanation of the Effect of Cholesterin in the Flocculation Reactions

It has already been shown (Eagle, 1930, 2) that the flocculation of antigen by syphilitic serum is in all respects analogous to bacterial or red cell agglutination, or foreign protein precipitation by an antibody serum. The reagin globulin of syphilitic serum is deposited as a film of denatured protein around the individual lipid particles, with a much higher critical potential than the underlying lipid antigen (10 to 15 millivolts instead of 1 to 5), and therefore much more sensitive to the flocculating action of electrolytes. Relatively small quantities of NaCl ($N/20$) suffice to depress the protein-coated particles below this critical value, whereupon they can cohere. The diagnostic criterion is the formation of either optically visible aggregates (Kahn, Sachs Georgi) or of a single cohesive floccule (Meinicke Klarungs reaction, Hinton agglutination test, Müller Ballungs-reaction, Lentochol reaction, etc.).

The coarsened dispersion state of the antigen already shown to be caused by cholesterin completely explains its effect in increasing the sensitivity, *i.e.*, the velocity of this aggregation.

1. The first and probably most important factor is the increased avidity of large particles of antigen for reagin, as already discussed in connection with the Wassermann reaction (Section III).

2. *Effect of Cholesterin Bulk.*—The individual particles of the antigen-cholesterol sol are, to all intents and purposes, pure antigen. The cholesterin core, masked by the antigen surface, acts as an inert filler, replacing just so much antigen. Adding cholesterin 0.5 per cent to an antigen already containing $1\frac{1}{2}$ per cent solid is equivalent to increasing the antigen content to 2 per cent. This would result in a correspondingly more rapid flocculation (Eagle, 1930, 4), partially explaining this sensitizing action of cholesterol.

3. *Effect of the Size of Particles upon the Velocity of Their Aggregation.*—It is obvious that the more finely divided a given quantity of

antigen, the more difficult is the formation of optically visible aggregates. In other words, a coarser dispersion would correspond to a preliminary quasi-aggregation, facilitating by just so much the subsequent aggregation by syphilitic serum. Moreover, with coarser particles there is less surface, and more reagin per unit surface, which also increases the velocity of aggregation (Eagle, 1930, 4).

Qualitatively, therefore, cholesterol would accelerate flocculation because it causes a coarser dispersion of antigen. The quantitative evaluation of this effect must await the ultramicroscopic measurement of the particle sizes in an antigen, as contrasted with an antigen-cholesterin, sol. Thus, if the average particle in an antigen sol measures, *e.g.*, 0.05μ radius, while the average particle formed by a cholesterolized antigen measures 0.5μ , an optically visible aggregate of, say, 2μ radius would necessitate the cohesion of 6×10^4 particles of the one, and only 64 of the other.

The foregoing applies with even greater force to those reactions in which the diagnostic criterion is not just visible aggregation, but sedimentation (Müller, Lentochol, etc.). The velocity of sedimentation is proportional to the square of the radius of the aggregate: and the larger the initial particles the fewer need aggregate in order to produce rapid sedimentation.

To summarize, the effect of cholesterol in increasing the sensitivity of antigen in the flocculation tests is due to many factors, the relative importance of which it is difficult to evaluate. They are listed below in what is considered to be the order of increasing importance.

1. Cholesterol acts as just so much added antigen, known to increase the sensitivity of flocculation.

2. With fewer and larger particles, there is less surface per unit antigen and therefore more reagin per unit surface, with a corresponding increase in the velocity of flocculation.

3. The coarse dispersion of antigen caused by cholesterol facilitates subsequent aggregation. Fewer particles need cohere to form visible or sedimenting clumps.

4. Large particles such as those seen in an antigen-cholesterin sol have an enormously greater avidity for reagin than the small particles formed by antigen alone.

V. SUMMARY AND DISCUSSION

When cholesterinized antigen is dropped into an excess of water, the rapid flocculation of cholesterin crystals is prevented by the fact that, as tiny aggregates form, they adsorb a protective surface of hydrophilic lecithin (*i.e.*, antigen) which endows the particles with its own stable surface properties and thus prevents further aggregation. The colloiddally dispersed antigen-cholesterin particles have approximately the same isoelectric point (pH 1.9), critical potential (1 to 5 millivolts) and coagulation value (0.75 M NaCl) as pure antigen particles of the same concentration, while the corresponding values for cholesterin are pH 2.1 to 3.4 (probably due to an associated impurity), >100 millivolts, and <0.001 N NaCl, respectively.

Presumably, this adsorption of antigen by the cholesterin nucleus is determined by the fact that the former has a lower surface tension against water. At any rate, many surface active substances (serum; alcoholic extract of milk, egg or any animal tissue; Na-oleate; Na-glycocholeate; Na-taurocholate) cause a similar stable dispersion of cholesterin; and conversely, many otherwise water-insoluble substances of the most diverse chemical structure can be made to form a stable colloidal suspension by adding antigen to their alcoholic solutions before dropping into water.

The colloidal suspension formed by antigen alone is very finely dispersed: only a few of the particles exceed the limits of dark field visibility. Cholesterin causes a marked increase in the number of these particles, out of all proportion to its mass; thus, one part of cholesterin to five of antigen causes a ten-fold increase in such visible particles, at the expense of the submicroscopic micellae formed by antigen alone. At the same time, the suspension becomes much more turbid. The particles remain discrete until the cholesterin: antigen ratio exceeds 1:1, when slight microscopic aggregation is observed; microscopic flocculation is seen only when this ratio exceeds 5:1, when there is not sufficient antigen to act as an efficient protective colloid.

Cholesterin therefore causes a coarsened dispersion of antigen by forming a relatively large nucleus upon which antigen is adsorbed. As shown in the text, the larger the antigen particle the greater is its avidity for reagin per unit surface or mass. Thus, the coarse sol formed

by dropping water-into-antigen is about twice as efficient as a finely dispersed antigen-into-water sol of the same concentration. The coarsened dispersion caused by cholesterol completely explains the greater sensitivity of the cholesterolized antigen in complement fixation.

The same factor obtains in the flocculation reactions. In addition, the coarsened dispersion acts as a preliminary quasi-aggregation, facilitating by just so much the subsequent formation of visible clumps (or sedimenting aggregates) upon the addition of syphilitic serum; moreover, there is less surface in a coarse sol, with more reagin per unit surface, and correspondingly more efficient flocculation.

The foregoing would be of purely academic interest were it not for the following considerations. From several points of view cholesterol is unsatisfactory as a sensitizer for antigen. Its solubility in alcohol is small. Even the 0.6 per cent concentration used in the Kahn test is difficult to keep in solution. Yet, as our experiments show, its sensitizing action increases indefinitely with its concentration. If it were sufficiently soluble, even 3 per cent could be used to advantage, increasing the sensitivity of $1\frac{1}{2}$ per cent antigen for complement fixation some 200 to 400 per cent, instead of about 50 per cent, as does 0.2 per cent cholesterol.

Since, as we have shown, the sensitizing action of cholesterol upon antigen is due solely to the coarse dispersion it causes, and since it is quite inert during the actual combination of the lipoid particles with reagin, it can be replaced by any substance with similar physical properties. The problem in hand was therefore to find a water-insoluble substance, very soluble in alcohol, with so high an interfacial tension against water that, as in the case of cholesterol, microscopic particles would adsorb antigen when the alcoholic solution of the two is dropped into water. Given such a substance, it would be possible to obtain a more sensitive antigen for both complement fixation and flocculation, but particularly for the former.

These theoretical expectations have been realized in a group of substances shortly to be reported: they make possible an antigen which is from 2 to 10 times as efficient in the Wassermann test as any now available.

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THE KILLING OF COLON BACILLI BY X-RAYS OF DIFFERENT WAVE LENGTHS

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The experiments to be described in this paper are quantitative studies of the rate at which colon bacilli die when subjected to known intensities of X-rays of various wave lengths. Such measurements are of interest for several reasons. They provide a more or less accurate description of the course of this biological reaction and they throw a definite, though as yet an indirect, light upon the mechanism by which these cells are killed. At the same time they provide an essential basis for attempts to alter cells without killing them and they give data which have a direct bearing upon the validity of the generally accepted principles of X-ray dosimetry as applied to tumor and other biological irradiations.

The biological¹ and physical² procedures are the same as those previously described. A few single bacilli (*ca.* 200 to 300 per square inch) were spread as evenly as possible upon the surface of an agar plate and two equal areas were stamped upon it. One of these areas was immediately given a measured dose of X-rays, the other served as a standard. The ratio of the counts made after incubation of the number of colonies growing out in the irradiated and standard regions is the survival ratio. In any one experiment the X-ray intensity was held constant and exposures of enough different lengths of time were made to determine the course of the survival ratio-dosage curve. In order that a sufficient number of bacteria should be counted to make the results statistically significant, at least ten plates were irradiated for each of the chosen exposure times and their results averaged. In this way each final survival ratio is the result of counts of not less than 2000 to 3000 organisms. Though the quantities thus obtained are called survival ratios,

¹ Wyckoff, R. W. G., and Rivers, T. M., *J. Exp. Med.*, 1930, 51, 921.

² Wyckoff, R. W. G., *J. Exp. Med.*, 1930, 52, 435.

they are in reality measures of the destruction of the reproductive function in the bacteria. It is possible, particularly with the softer radiations, that this loss of ability to divide takes place without cell death.

The previously described³ gas tube, equipped with targets of different metals, has been used to obtain the intense beams of X-rays needed for these experiments. In this way, killing rates upon *B. coli* have been measured for X-ray beams consisting mainly of the K lines of silver, molybdenum, copper and chromium and the L lines of silver. Because of the loss of intensity consequent upon filtration and the desirability of having the data with the different radiations as comparable as possible, no attempt has been made to render the beams in any of these experiments more nearly monochromatic. The earlier experiments with filtered and unfiltered copper rays indicate, however, that only a moderate error is introduced by this heterogeneity.

The current through the X-ray tube during the irradiations was held constant at either 4 or 8 milliamperes. The peak voltage across it depended on the target—for Ag K, Cu K and Cr K rays it was 34 KV, for Mo K it was 34 or 38 KV and for Ag L, 21 KV as measured with a 12.5 cm. sphere gap. The beam intensity was determined using the small air ionization chamber described in the earlier experiments with copper rays. The molybdenum and silver K radiations are sufficiently hard so that this air ionization was taken as a direct measure of the X-rays striking the irradiated agar surface. With the other and softer rays, correction was made for the amount absorbed in the layer of air between the surface of irradiation and the volume giving the ionization current. The length of this air column was 3.2 cm. Taking the absorption coefficient⁴ of Cu K radiation as $\mu/\rho = 8.43$, of Cr K as 28.0 and Ag L ($\lambda = 3.98 \text{ \AA}$) as 129.3, calculation shows that the measured ionizations are 97 per cent for Cu K, 89 per cent for Cr K and 59 per cent for Ag L of those due to the X-rays at the irradiated surface. Introducing this absorption correction and transferring to standard air conditions, the measured air ionizations of the beams used in the experiments of Table I are recorded in this same table as the saturation currents due to the ions produced in a cube 1 cm. on its edge.

Typical experiments giving survival ratios with each of the foregoing radiations are reproduced in Table I. The ratios thus obtained, as well as those from several similar experiments, are plotted on a semilogarithmic scale in Figs. 1 to 5. It is evident that the results with all radiations lie on straight lines in these diagrams.

³ Wyckoff, R. W. G., and Lagsdin, J. B., *Radiology*, 1930, 15, 420.

⁴ Kaye, G. W. C., X-rays, London, 1923, 4th ed., 145; Jönsson, E., Uppsala Univers. Arsskrift, 1928.

TABLE I
Survival Ratios for Various Radiations

Irradiation times	Survival ratios for				
	Ag K (0.564A)	Mo K (0.710A)	Cu K (1.537A)	Cr K (2.29A)	Ag L (3.95A)
<i>seconds</i>					
5	—	—	0.714	—	—
10	—	0.863	0.687	0.629	—
20	0.676	0.693	0.512	0.392	0.689
30	—	0.612	0.277	0.293	—
40	0.443	0.491	0.220	0.185	0.518
50	—	—	—	0.128	—
60	0.334	0.380	0.149	0.100	0.322
80	—	0.300	—	—	—
90	0.192	—	—	—	0.263
120	0.124	—	—	—	0.173
Ionization per sec./cm. ² in 1 cm. of air	73.1	94.1	173.1	274.5	133.8 e.s.u.

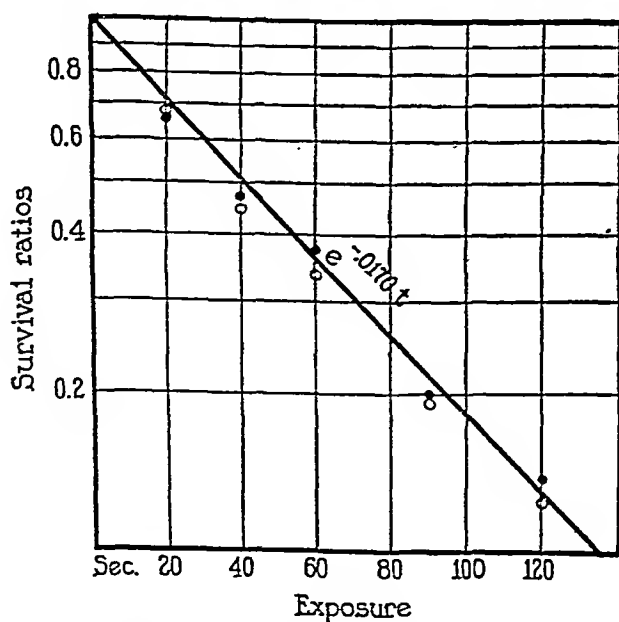


FIG. 1. Data from two sets of experiments with a tube giving silver K radiation. The ratios of Table I are plotted as the open circles.

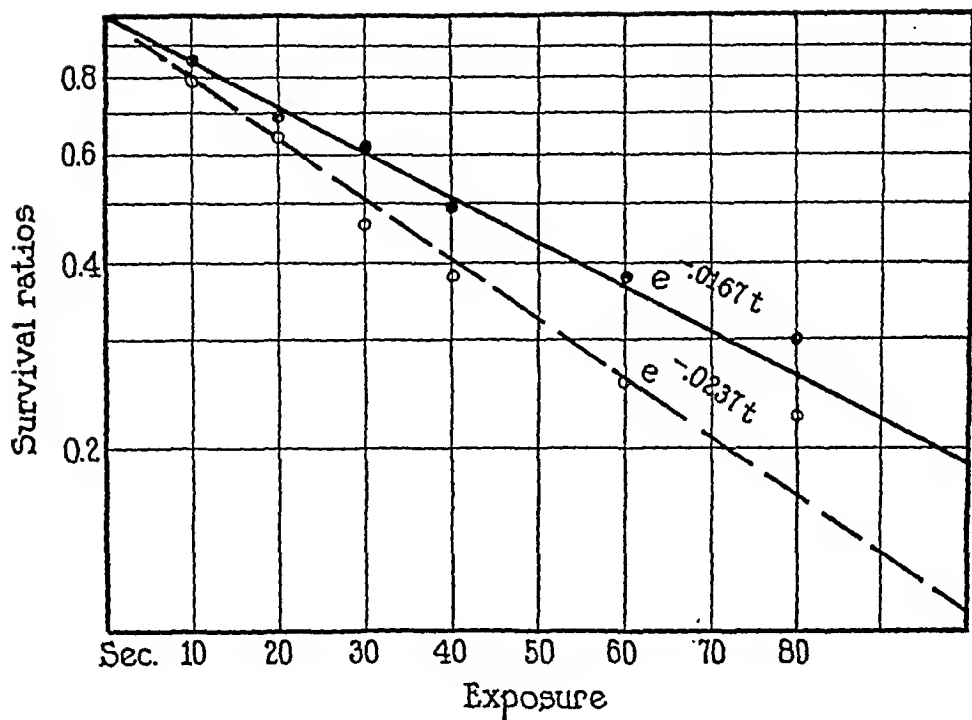


FIG. 2. Data from two different sets of experiments with a tube giving molybdenum K radiation. The ratios of Table I are plotted as full circles.

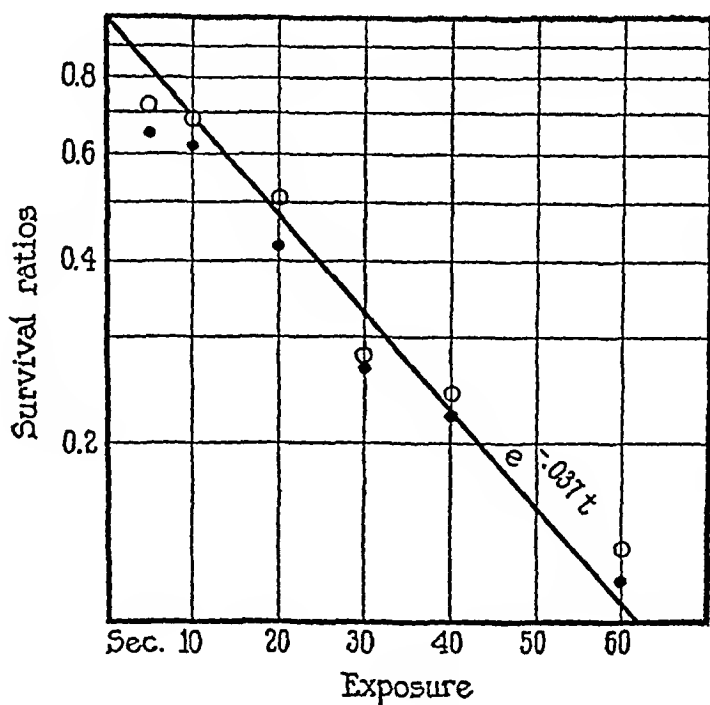


FIG. 3. Data from two sets of experiments with a tube giving copper K radiation. The ratios of Table I are plotted as open circles.

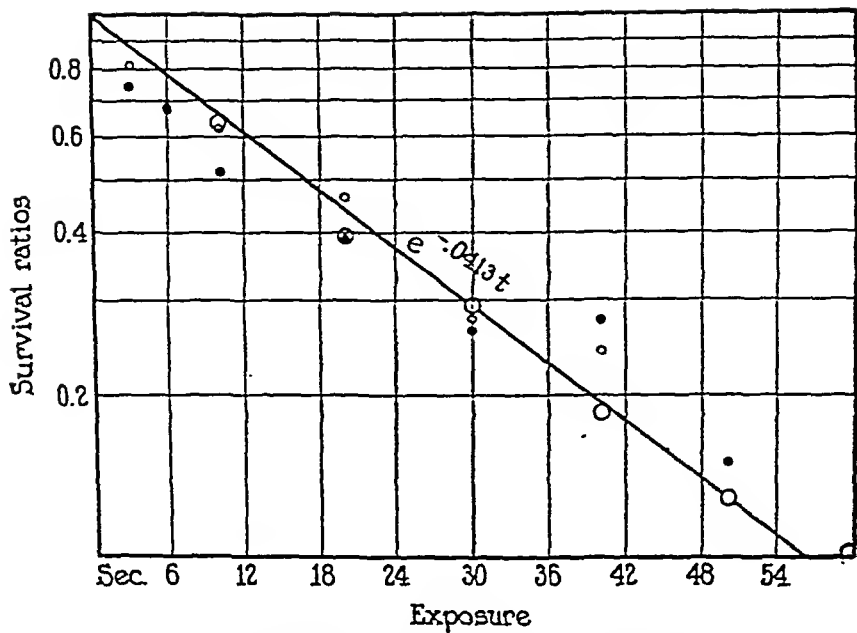


FIG. 4. Data from three sets of experiments with a tube giving chromium K radiation. The ratios of Table I are plotted as the large open circles.

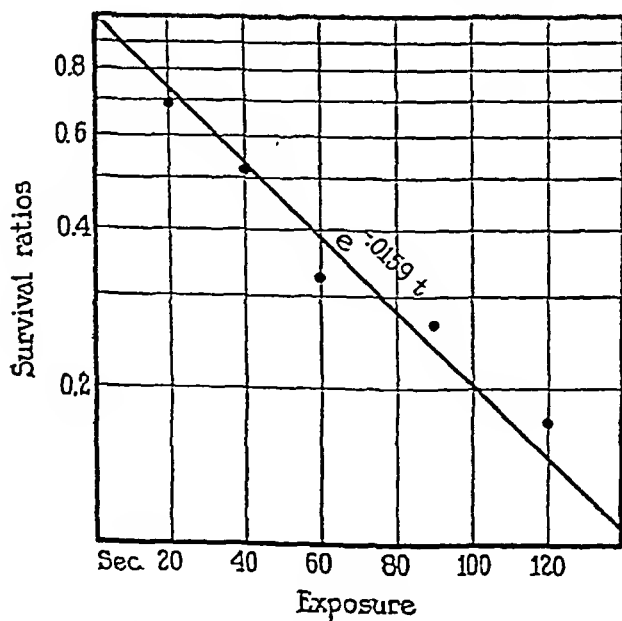


FIG. 5. Data from the set of experiments with a tube giving silver L radiation that is recorded in Table I.

Analysis

The same analysis based upon the quantized absorption of X-rays which was outlined previously for copper rays is applicable to all these experiments. From this standpoint the straight lines of the figures are proof that the absorption of one quantum of X-ray energy is sufficient to produce death even with the very soft silver L radiation. This agrees with the results of Holweck⁵ and Lacassagne⁵ with *B. pyocyaneus*. As before, the average or expected number of absorptions per bacterium can be computed for each experiment from the beam intensity, the size of the bacterium and a knowledge of the number of ions liberated in air by one quantum of the wave length used. A typical calculation⁶ showing the quantities employed throughout has already been outlined. The expected numbers of absorptions, computed for the several wave lengths of this series of experiments, are listed as "Calculated α " in Table II. Since the survival ratios give straight lines when plotted semilogarithmically, they must be expressed by an equation of the type

$$A_1/A_0 = \text{survival ratio} = e^{-\alpha'} \dots\dots\dots (1)$$

The α 's that are given by the slopes of the lines of Figs. 1 to 5 are listed in Table II. If every quantum absorbed were deadly, these α 's would equal the calculated values of α . Since they are much smaller, their ratio, α/α' , furnishes the average number of absorptions which occur before a bacterium is killed. If the volume through which a quantum acts were small compared to that of the vital parts of a cell, then the reciprocal of this quantity, or α'/α , would measure the volume of the vital elements. Physical experiments indicate that this is not the case, however, and as a consequence the ratios α'/α , at least for the harder X-rays, are more an index of the size of the quantum's sphere of action than of the bacterium's vital elements.⁷

⁵ Holweck, F., *Compt. rend.*, 1929, 188, 197; Lacassagne, A., *Compt. rend.*, 1929, 188, 200.

⁶ Wyckoff, R. W. G., *J. Exp. Med.*, 1930, 52, 435.

⁷ It is interesting to note in this connection that the curve showing the increase in sensitive volume with harder rays is very similar to curves that are obtained when the range (in air) of electrons is plotted against their speed (*cf.* the data of Lenard and of Wilson quoted by H. Küstner in *Ergebnisse d. med. Strahlen-*

Nevertheless, it can with safety be concluded that these cell constituents which are essential to its life, or at least to its growth through multiplication, cannot in their sum total have a greater relative volume than α'/α . The "sensitive volumes," α'/α , furnished in this way by the several radiations studied are given in Table II. Together with their reciprocals they are shown graphically in Fig. 6. Since α'/α can be as small as 0.014 (for Ag L radiation), the region within a colon bacillus which must be injured to prevent cell multiplication cannot be more than this fraction of the bacterial volume. The manner in which the curve of Fig. 6 flattens out with increasing wave length suggests that the true sensitive volume of this organism may not be far from 0.01. These experiments cannot show whether

TABLE II

	Ag K	Mo K	Cu K	Cr K	Ag L
Calc. α	0.079	0.126*	0.527	1.220	1.108
Obs. α'	0.0174	0.0167*	0.0370	0.0413	0.0159
Sensitive volume $\frac{\alpha'}{\alpha}$	0.220	0.159	0.0702	0.0338	0.0144
Hits to kill $\frac{\alpha}{\alpha'}$	4.54	6.46	14.2	29.5	69.7
β in expression (2).....	0.0238	0.0215	0.0231	0.0150	0.0119

* These numbers refer to the experiment of Table I; the remaining results for molybdenum are averages of more than one experiment.

the sensitive part is a single structure or is the sum of a number of smaller vital elements. If, however, as appears natural, it is associated with the chromatin material of the cell, then the latter is probably true.

The sensitive volume as calculated above possesses a large possible error. At least two factors contribute to this potential inaccuracy. One is the non-monochromatic character of the X-ray beams used, the other and more important lies in the serious physical problems met in measuring such an intense beam of soft X-rays. Consistent readings of intensity have been obtained but extended research is nec-

forschung, Leipzig, 1925, Band I, 229. This suggests that the biological sphere of action of an absorbed quantum is less than the spherical volume within which it liberates all its ions.

essary before their absolute accuracy is satisfactorily known. The previously published results with Cu K rays are in moderate agreement with those of this paper. Most of the difference which exists is, how-

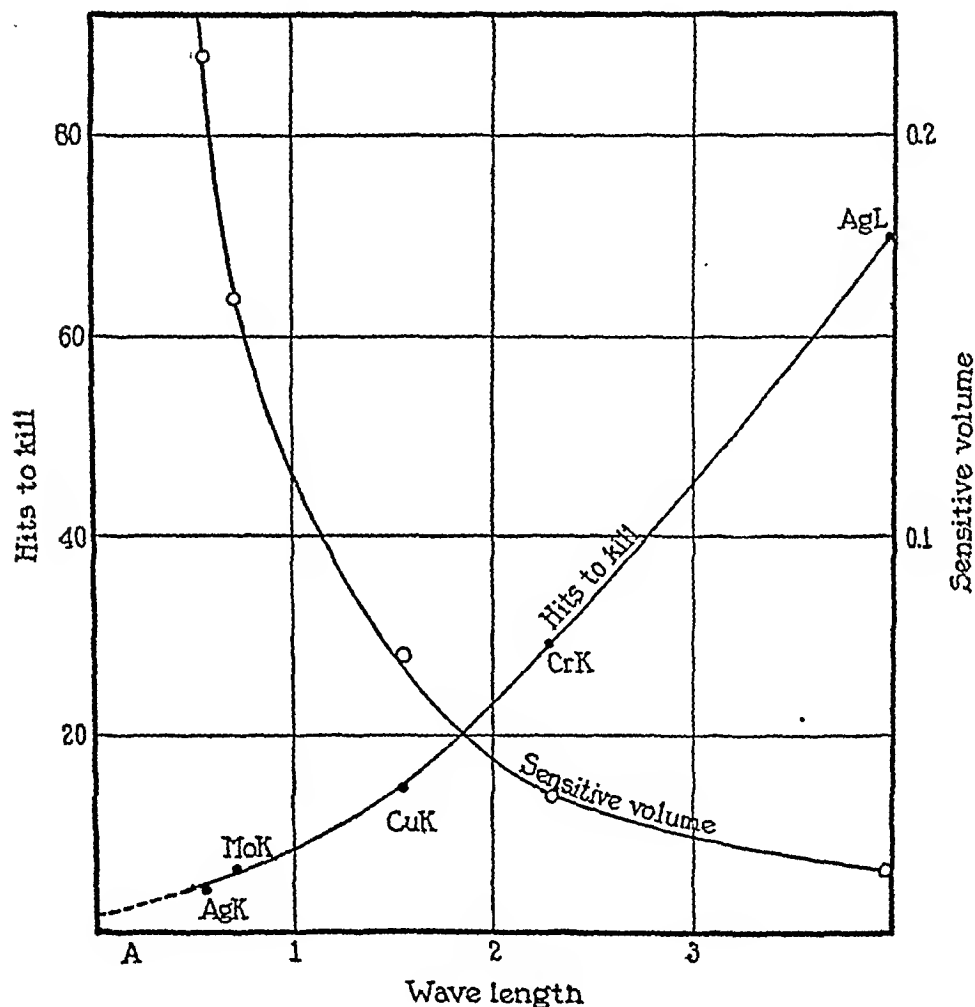


FIG. 6

ever, to be ascribed to improvements which have been made in the measurement of beam ionization.

It is also instructive to state the killing action of these different wave lengths in terms of the energy rather than the quanta absorbed. This

can readily be done through the measured air ionization using an equation of the form

$$A_1/A_0 = e^{-\beta u} \dots\dots\dots (2)$$

where u is expressed in electrostatic units per second per cubic centimeter of air under standard conditions (in roentgen units). The exponents β as found for the wave lengths used are given in Table II. If the killing were proportional to the number of ions produced (in air and undoubtedly also in the bacteria) these β 's should be constant. For the very soft Cr K and Ag L radiations they are definitely smaller than for shorter X-rays. If the same amount of energy is consumed in producing an ion pair in air for all these radiations, as the best available information⁸ indicates to be true, then this result must mean that more energy is needed to kill a bacterium when the quantum is small.

DISCUSSION

Throughout the analysis which has been followed in interpreting these experiments, it has been considered that differences which might exist in the resistances of individual organisms to X- and cathode rays were not important. With the high velocity electrons of a cathode ray beam where nearly every electron that is absorbed brings about death, this conclusion is a necessary one. It is a strong argument in favor of its continued applicability that the killing rate of bacteria is the same for these electrons and for X-rays throughout the range of wave lengths studied.

The statement is commonly encountered that whenever bacteria are subjected to an unfavorable environment, be it physical or chemical, they die off at a rate which is logarithmic for at least part of the time. By many it has been thought that such a decrease in bacterial count meant that death of these cells was a monomolecular chemical reaction; others have asserted that these curves of death are indices, not of the mechanism of killing, but rather of the relative sensitivities of the bacteria in the cultures used. The comparative merits of these two points of view have been discussed frequently but not conclu-

⁸ Kulenkampff, H., *Ann. d. Physik*, 1926, 79, 97.

sively.⁹ If relative sensitivities were the predominant factor, then it might be expected that the death-rate curves for a particular organism would be much the same irrespective of the killing agent. In making such comparisons the entire curve and not merely its middle portion is significant. When this is taken into account the curves for the action of different physical agents are often found to be of unlike shape and are not necessarily semilogarithmically linear. Thus experiments on the death-rates of spores and of some actively metabolizing bacteria indicate that these rates depend upon both the environment and the type of organism. The curves of some of these rates¹⁰ seem semilogarithmically linear; others¹¹ depart widely from such a relation.

Few of the studies of the effects of radiations on cells give useful data. The death-rate of staphylococci when subjected to ultraviolet light of a single wave length has been measured.¹² These results, plotted on a suitable semilogarithmic scale (Fig. 7), give a curve which is not as straight as the line obtained when this bacterium is irradiated with cathode rays but is nevertheless of a smooth multiple-hit type. Lacassagne and Holweck have found that the death-curves of *B. pyocyaneus* are straight if killing is effected with Ag L rays but not with the still softer Al K radiation. These facts are opposed to the idea that the killing-curves with which they are concerned are primarily expressions of relative sensitivity.

Though it is quite clear that this factor of "biological variation" cannot be of predominant importance in either the X-ray experiments with *B. pyocyaneus* or in these observations on *B. coli*, no conclusive data exist to prove that it does not occur as a secondary factor to influence the foregoing analysis. The experiments bearing most directly on this problem are the recent observations of Gates¹³ showing that except possibly in strongly alkaline solutions the survival ratios of *S. aureus* irradiated by ultraviolet light are independent of the pH of the medium in which they grow. Since death-curves with heat are known

⁹ See for example papers mentioned in Rahn, O., *J. Gen. Physiol.*, 1929, 13, 179.

¹⁰ Bigelow, W. D., *J. Inf. Dis.*, 1921, 29, 528.

¹¹ See the chart reproduced by Esty, J. R., in Jordan, E. O., and Falk, I. S., *The New Knowledge of Bacteriology and Immunology*, Chicago, 1928, 299.

¹² Gates, F. L., *J. Gen. Physiol.*, 1929, 13, 231.

¹³ Gates, F. L., *J. Gen. Physiol.*, 1929, 13, 249.

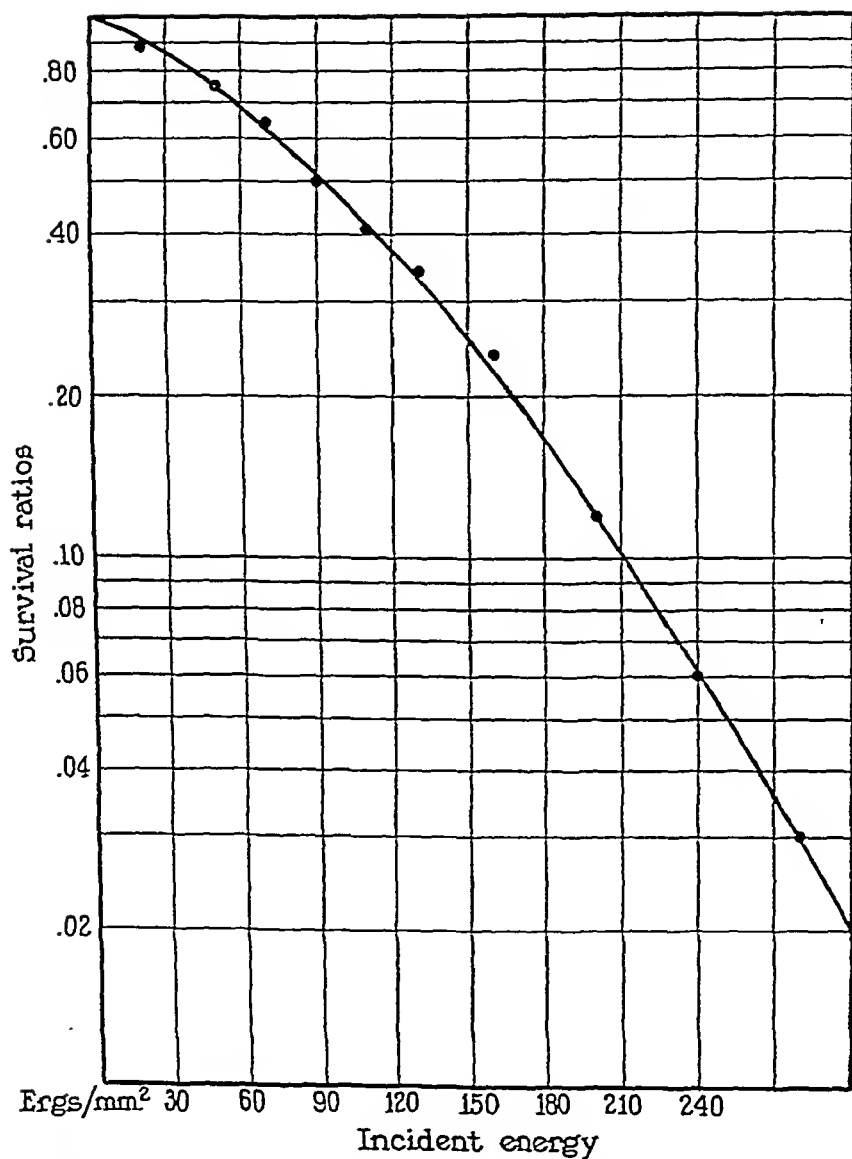


FIG. 7. A plot of the data of Gates showing the killing rates of *Staph. aureus* for light of wave length $\lambda = 2540 \text{ \AA}$.

to be influenced by the acidity of the medium,¹⁴ this is strong evidence that killing rates with ultraviolet radiation are not concerned with the sensitivity of the organisms as measured by some other physical agents. It is to be expected that sensitivity should be even less important with X-rays.

In so far as results with a relatively small bacterial cell are applicable to large tissue cells, the measurements of the amount of ionization necessary to produce death for different radiations have a bearing on the principles of X-ray dosage. The general constancy of β for the harder rays of Table II is in good agreement with the assumption that ionization, as determined by an air chamber, is an accurate measure of the biological action of the rays. The smaller β 's found for very soft rays are, on the other hand, an indication that air ionization data with a Bucky "grenz-ray" tube, for example, are probably not, from a biological standpoint, accurately comparable with those from the more ordinary types operated at higher voltages.

Valuable help in carrying out these experiments has been given by Charles G. Porskieves.

CONCLUSIONS

X-ray beams of wave lengths lying in the range between 4 Å and 0.5 Å all kill *B. coli* in a semilogarithmically linear fashion. Interpreted in terms of the known quantized absorption of X-rays, this means that one absorption of any of these radiations is sufficient to kill. Though death results from a single absorption, only about one hit in four with Ag K and one in sixty with Ag L radiation is deadly. The course of curves constructed from these experimental results suggests that the portion of this bacterium which is essential to its continued life has a total of approximately 0.01 of the cell volume.

For copper and harder radiations the biological action of the rays is proportional to their measured air ionization. The same biological change with the softer chromium K and silver L X-rays seems to require a somewhat more intensely ionizing beam.

¹⁴ Bigelow, W. D., *op. cit.*

THE EFFECT OF BACTERIAL VARIATION UPON THE FACTORS NECESSARY FOR THE PHENOMENON OF LOCAL SKIN REACTIVITY TO BACTERIAL FILTRATES

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If an injection of a bacterial filtrate into the skin of a rabbit is followed 24 hours later by an intravenous injection of a culture filtrate of the same or another bacterium there appears an extremely severe hemorrhagic necrosis at the prepared skin sites. This phenomenon of local skin reactivity was described by the present author (1-12) and corroborated by Hanger (13), Ecker and Welch (14), and Frisch (15). Among the various phases of the phenomenon thus far studied there has been reported the specific neutralizability of the reacting factors (*i.e.*, intravenous factors) by homologous immune sera (9, 10). The object of the work presented in this paper was to study the serum neutralizability of reacting factors derived from various stock strains of the same bacterium and also to determine the influence of bacterial variation upon the serum neutralizability of these factors.

EXPERIMENTAL

Horse and rabbit immune sera were employed in this work. The immunization of rabbits was carried out by semi-weekly simultaneous subcutaneous and intravenous injections. Filtrates of 6 day old cultures in tryptic digest broth were injected subcutaneously. Unwashed heat-killed vaccines were injected intravenously. The doses were increased by 25 percent at each injection. The injections were carried out for 4 to 6 weeks. 25 cc. of the filtrate was the largest subcutaneous dose and 20 cc. of the vaccine (1 billion organisms per cubic centimeter) was the largest intravenous dose. In attempting to prepare sera against *B. typhosus* rough variants and against a strain of *B. typhosus* passed through mice (Ty 159 Mouse 46), considerable difficulties were encountered because of the high mortality rate of the rabbits under treatment. The procedure of immunization was then modified for these strains, as follows:

The initial dose of vaccine was 100,000,000 organisms. The largest dose given

at the last injection was 5 billion organisms. Instead of filtrates of tryptic digest broth cultures, filtered washings of 24 hour old cultures on solid media were used for subcutaneous injections. The first dose was 0.1 cc. The largest dose was 3 cc.

Horse sera were prepared according to the method described in a previous publication (9). The length of time during which the immunization was continued before each bleeding is indicated in Table II.

The *B. typhosus* strains used for the experiments about to be described were designated Ty T_L stock, Ty 157 stock, Ty 159 stock, Ty 864 stock, Ty 870 stock and Ty 240 stock. The *B. coli* strains also employed for some experiments were called C 42 and C stock. These strains were kept in the laboratory collection for from 1 to 4 years prior to this work.

The colony appearance of these strains on plain agar, with the exception of Ty T_L stock and C 42 stock, was of the normal smooth type. The Ty T_L stock strain showed colonies which appeared distinctly granular under the low dry power. Their margin was ameboid and somewhat ragged but this was not sufficiently pronounced to classify this strain as rough. This intermediate type of roughness was, however, a stable characteristic which persisted during the entire year of observation without any change either in the direction of smoothness or more pronounced roughness. This stability was ascertained by 42 "platings" of this strain. No typical rough colonies were obtained from old fluid cultures.

The *B. coli* strain C 42 stock, kindly sent to me by Dr. André Gratia 6 years ago, was a typical rough strain obtained by him from a bacteriophage lysed culture. The colonies were cauliflower and coarsely granular.

Broth cultures of the various strains, with the exception of Ty T_L stock and C 42 stock strains, gave uniform growth after 24 hours of incubation at 37.5°C. The Ty T_L stock strain produced, in addition to the uniform cloudiness, an insignificant amount of precipitate which on shaking gave rise to easily broken up flakes. The C 42 stock strain grew only on the bottom of the test tube, the supernatant broth remaining perfectly clear.

All the *B. typhosus* stock strains showed well pronounced motility.

24 hour cultures of various strains on plain agar were washed in distilled water by centrifugalization and resuspended in 0.85 per cent and 1.4 per cent NaCl solution. The suspensions were incubated in a water bath at 37.5°C. for 2 hours and then placed in the refrigerator for 22 hours. The C 42 stock suspension was completely agglutinated but the remaining strains stayed in suspension.

The various strains were grown in immune sera in order to obtain rough variants. The results were as follows:

12/2/1929, the Ty T_L stock strain was inoculated into plain broth containing 1-10 dilution of Serum R-238 (homologous antityphoid rabbit serum). After 24 hours of incubation at 37.5°C. the growth appeared clumped. The culture was further incubated at room temperature until 12/17/1929. At this date it was plated out on plain agar. The colonies appeared typically rough. 12/18/1929, a rough colony was fished into broth containing 1-10 dilution of Serum R238 and

incubated at 37.5°C. for 24 hours and later at room temperature. 1/17/1930, the culture was plated out on plain agar and a single colony which appeared distinctly rough was transplanted onto a plain agar slant. The rough strain thus derived from the Ty T_L stock strain was designated Ty T_L 238 ser₂ strain. This strain grew in plain broth in flaky clumps leaving the supernatant broth perfectly clear; it gave complete agglutination in 0.85 per cent and 1.4 per cent NaCl solutions and was non-motile. The fermentation of sugars was typical of *B. typhosus*. The Ty T_L 245 ser₂ strain was prepared from the Ty T_L stock strain in an identical manner except that Serum R245 was used (homologous antityphoid rabbit serum). The strain was also typically rough as ascertained by the appearance and above described tests. Antityphoid bacteriophage, obtained from a convalescent typhoid patient in the usual manner, was also employed in order to induce bacterial dissociation, as follows:

1/15/1930, a tube of broth containing 1-10 dilution of the bacteriophage was inoculated with a young culture of Ty 870 stock strain, incubated for 24 hours at 37.5°C. and at room temperature for 48 hours and then seeded onto a plain agar plate. The colonies which appeared rough were fished into broth containing 1-10 dilution of the same bacteriophage and again incubated as above until 1/22/1930. At this date a third passage of a rough colony was made through the bacteriophage. The culture plated out (1/29/1930) gave a number of distinctly rough colonies. A culture on plain agar derived from a single colony was designated Ty 870 phage₃. The strain persistently retained its roughness as ascertained several times by the tests in plain broth, NaCl solutions, and unchanged appearance of growth on solid media.

B. coli C stock strain was converted into a rough strain by means of seven daily passages through 10 per cent Serum H₁₄₈ (anti-*coli* horse serum) broth. The 8th passage yielded typically rough colonies, as proven by the above described tests. This strain was designated C rough.

The other strains of *B. typhosus* employed here did not undergo any dissociation in spite of persistent attempts, as shown in the following protocols:

Ty 864 stock was passed 12 times through 1-10 dilution of antityphoid horse serum in plain broth. The culture of each passage was incubated for 24 hours at 37.5°C. and 9 days at room temperature, and plated out on plain agar at the end of this period. A single colony appearing rough was used for the next passage. Four passages yielded colonies which appeared questionably rough. The colonies, however, promptly reverted to the normal appearance when subcultured on plain agar for several generations. The same strain similarly passed through the antityphoid bacteriophage did not undergo any change. The other two strains of *B. typhosus* employed in this work (*i.e.*, Ty 159 and 240 stock) were also passed a number of times through antityphoid immune horse and rabbit sera and antityphoid bacteriophage. No dissociation was accomplished in spite of a large number of passages.

The various *B. typhosus* stock strains were also passed through mice in order

to increase the virulence of the strains and possibly enhance their smoothness. The technique adopted for the purpose was, as follows:

A 24 hour old agar slant of a given strain was emulsified in 0.85 per cent NaCl solution to a turbidity representing approximately 1 billion organisms per cubic centimeter. 1 cc. of the emulsion was injected intraperitoneally into 2 mice. 24 hours later cultures were made on Endo agar plates from the heart's blood of the dead or killed mice. The 24 hour old growth on the plates was suspended to the above indicated turbidity in 0.85 per cent NaCl solution. One half of 1 cc. of the emulsion was used for injection into a mouse. Only heart's blood cultures were employed for passages. The strains passed through mice were designated mouse strains, with the name of the stock strain from which they were derived and a number indicating the number of passages. None of the stock strains (including the Ty T_L strain) underwent any change in the appearance of the colonies in spite of numerous passages.

The different stock strains of *B. typhosus* and *B. coli*, the variants obtained by means of immune sera and bacteriophage and the strains passed through mice were utilized as follows:

I. Serum Neutralizability of B. typhosus and B. coli Reacting Factors

a. Preparation of Toxic Filtrates.—The toxic filtrates necessary for the phenomenon of local skin reactivity were "agar washings" filtrates prepared in a manner previously described (6, 7). These filtrates were designated by their number and the name of the strain employed for their preparation. The "mouse" strains did not undergo more than one passage through artificial media previous to their use for the preparation of toxic filtrates. The 24 hour old heart's blood cultures on Endo agar plates were seeded into plain broth. The plain broth properly diluted served as the inoculum for the Kolle flask agar cultures necessary for the preparation of the filtrates.

b. Titration of the Reacting Factors.—The quantitative measurement of the reacting factors was carried out as follows:

The rabbits used for titrations were each injected intradermally with 0.25 cc. of the undiluted filtrate and divided into groups of four. 24 hours later a single intravenous injection of the filtrate diluted in 0.85 per cent NaCl solution was given to each rabbit. The dose was 1 cc. per kilo of body weight. Each group of rabbits received intravenously a different dilution of the filtrate. The local reactions were read 4 to 5 hours after the intravenous injections. The titrations were carried until the lowest dilution was found which gave no reaction in the 4 rabbits tested as well as the highest dilution which gave reactions in one or more rabbits of the group. The minimal dose of reacting factors was then considered as lying between these two figures. If a given filtrate was employed for any length of time, repeated control titrations were done. In these control tests, the dilutions employed were both the highest dilution capable of eliciting reactions and the lowest dilution giving no reactions. This was necessary since a loss of

potency was obtained during storage of filtrates (10, 11) as well as an occasional increase in the potency such as was recently observed.

The above described titration of the reacting factors is different from the method of titration previously described (9, 10, 12). In the older experiments the object was to demonstrate the serum neutralizability of the reacting factors and also to compare the neutralizing potency of various sera against one given batch of filtrate used throughout the work. For this purpose it was sufficient to determine the smallest amount of the filtrate capable of eliciting reactions in a high percentage

TABLE I
The Titer of Reacting Factors in Various Bacterial Filtrates

No. of filtrate	Strain used for preparation of filtrate	Highest dilution of filtrate eliciting reactions	Per cent positive rabbits with highest dilution eliciting reactions	Lowest dilution of filtrate showing no reactions	No. of reacting units per cc. of filtrate	Per cent of positive rabbits with various numbers of reacting units	
						6 reacting units	10 reacting units
A ₁₀	Ty T _L stock	1:400	50	1:600	500	—	—
A ₂₁	Ty T _L stock	1:800	50	1:1000	900	—	—
A ₃₁	Ty T _L stock	1:600	50	1:800	700	80	80
823	Ty T _L 238 ser ₁	1:600	50	1:800	700	—	—
778	Ty T _L 238 ser ₁	1:700	25	1:800	750	—	—
834	Ty T _L 245 ser ₁	1:600	50	1:700	650	—	—
989	Ty T _L 245 ser ₁	1:200	50	1:400	300	90	100
852	Ty 157 stock	1:1000	25	1:1200	1100	70	100
846	Ty 864 stock	1:400	25	1:600	500	—	—
848	Ty 159 stock	1:200	50	1:400	300	—	—
779	Ty T _L Mouse ₄₁	1:300	25	1:400	350	—	—
838	Ty T _L Mouse ₄₁	1:500	25	1:600	550	—	—
840	Ty 159 Mouse	1:550	25	1:700	625	—	—
971	Ty 159 Mouse ₄₁	1:1100	50	1:1400	1250	80	80
980	Ty 240 Mouse	1:700	25	1:900	800	—	—
911	Ty 870 phage ₂	1:700	25	1:900	800	—	—
1042	C stock	1:900	50	1:1100	1000	—	—
985	C rough	1:1000	50	1:1200	1100	—	—

of rabbits. However, in this work, in which studies on the neutralizability of various filtrates derived from different strains were planned, the method of titration as presented in this paper became necessary because of its sharper "end point." Table I embodies the titers of the reacting factors in various filtrates.

As is seen from Table I, all the stock strains tested, as well as their variants were capable of producing highly potent toxic substances.*

* It is interesting to note that provided one area of the skin is prepared the amount of reacting factors necessary to elicit reactions in the skin of certain rab-

As will also be seen from the same table the minimal dose of reacting factors produced reactions only in a small percentage of rabbits in the majority of instances. In planning the neutralization experiments about to be described the question arose as to what was the minimal number of reacting units which would yield a high percentage of reactions and, therefore, could be safely used for these experiments. Various multiples of the minimal doses were tested in groups of ten rabbits. As is seen from Table I, 6 and 10 units already gave a high percentage of reactions.

c. Serum Neutralization of Reacting Factors of Various Filtrates.—The technique employed for the neutralization experiments was similar to one described before (9, 10). One area of the skin of the abdominal wall of a rabbit was injected with 0.25 cc. of an undiluted filtrate. 22 to 24 hours later a single intravenous injection was given of a mixture of the same filtrate (diluted previously in 0.85 per cent NaCl solution to the desired degree) with a given undiluted serum in proportion 4:1. The mixtures prepared on the morning of the experiments were incubated in a water bath at 37.5°C. for 1 hour. The precipitate in the mixtures was broken up by shaking immediately before the injection. The intravenous dose of the mixture was 1.25 cc. per kilo of body weight. The readings of the reactions were made 4 to 5 hours after the intravenous injection. Each mixture was tested in 4 rabbits. If no reactions were obtained in the 4 rabbits tested, the result was considered as showing "consistent" neutralization of the reacting factors. If 1 or 2 out of 4 rabbits tested showed reactions the result was recorded "irregular" neutralization, provided not less than 6 minimal doses of the reacting factors were present in each 1.25 cc. of the mixture. This conclusion was fully justified since the control titrations with this amount of reacting factors showed from 70 to 90 per cent of positive rabbits (Table I). If 3 out of 4 or all rabbits tested showed reactions there was recorded "no neutralization."

Table II represents the results of neutralization experiments with various amounts of reacting factors of different toxic filtrates mixed with constant amounts of certain immune sera.

As is seen from Table II and Diagrams 1 and 2, two types of neutra-

bits may be amazingly small. In one instance, 0.0009 cc. of the filtrate was capable of eliciting a severe hemorrhagic necrosis at the prepared skin site. If one also considers that this amount was diluted in at least 100 cc. of rabbit's blood and that a part of it must have been lost in the tissues before reaching the prepared skin site, the amount actually capable of inducing the lesion is less than 0.000009 cc.

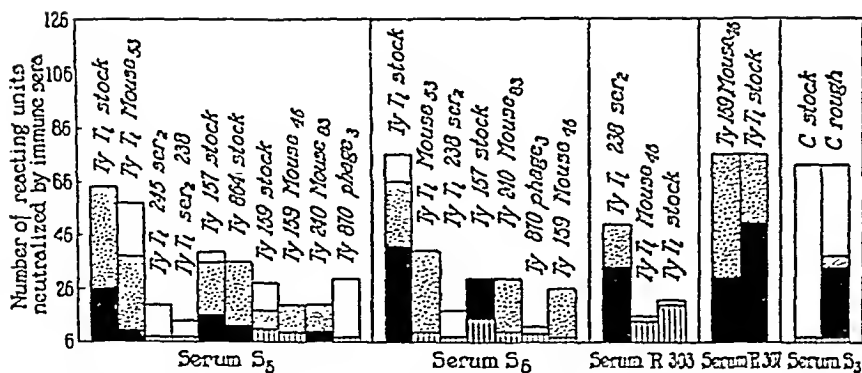


DIAGRAM 1

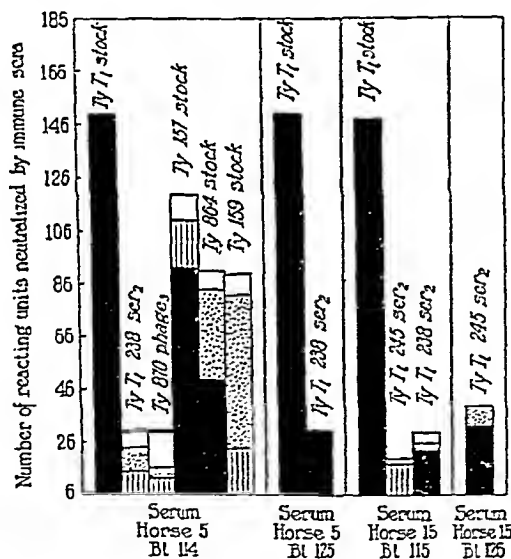


DIAGRAM 2

- - Not tested
- ▨ - Irregular neutralization
- - No neutralization
- - Complete neutralization

PHENOMENON OF LOCAL SKIN REACTIVITY

TABLE II
Serum Neutralizability of Various Bacterial Filtrates

No. of filtrate	Strain used for preparation of filtrate	No. of serum used for neutralization	Strain used for preparation of serum	Degree of neutralization of various amounts of reacting units by means of immune sera		
				Consistent neut.	Irregular neut.	No neut.
A ₂₆	Ty T _L stock	S ₆	Ty T _L stock	16, * 23, 24	30, 63	=
834	Ty T _L 245 ser ₂	S ₅	Ty T _L stock	—	6	11, 13, 19
823	Ty T _L 238 ser ₂	S ₆	Ty T _L stock	—	6	9, 12
839	Ty T _L Mouse ₂₃	S ₆	Ty T _L stock	9	10, 27, 38	58
852	Ty T _L 157 stock	S ₆	Ty T _L stock	13, 16	17, 20, 25, 33	40
846	Ty 864 stock	S ₆	Ty T _L stock	12	14, 20, 26, 35	=
848	Ty 159 stock	S ₆	Ty T _L stock	—	12	25, 28
911	Ty 159 Mouse ₁₆	S ₆	Ty T _L stock	—	9, 17, 20	=
980	Ty 870 phage ₃	S ₆	Ty 240 Mouse ₆₀	10	—	8, 10, 15, 30
A ₂₅	Ty 240 Mouse ₂₃	S ₆	Ty T _L stock	8.5, 12.5, 18.5, 26.5, 30	14, 20	=
823	Ty T _L 238 ser ₂	S ₆	Ty 240 Mouse ₆₀	—	55	75
852	Ty 157 stock	S ₆	Ty 240 Mouse ₆₀	15, 30	—	8, 12, 18
971	Ty 159 Mouse ₁₆	S ₆	Ty 240 Mouse ₆₀	—	8, 12, 18, 26	=
839	Ty T _L Mouse ₂₃	S ₆	Ty 240 Mouse ₆₀	—	10, 20, 40	=
980	Ty 240 Mouse ₂₃	S ₆	Ty 240 Mouse ₆₀	—	10, 20, 30	=
911	Ty 870 phage ₃	S ₆	Ty 240 Mouse ₆₀	—	—	=
989	Ty T _L 245 ser ₂	S ₆	Ty 240 Mouse ₆₀	—	50	10
971	Ty 159 Mouse ₁₆	R-303	Ty T _L R-238 ser ₂	12, 24, 36	—	=
A ₂₅	Ty T _L stock	R-303	Ty T _L R-238 ser ₂	—	—	15
A ₂₅	Ty T _L stock	R-303	Ty T _L R-238 ser ₂	—	—	20
971	Ty 159 Mouse ₁₆	R-307	Ty 159 Mouse ₄₀	25, 50	75	=
		R-307	Ty 159 Mouse ₄₀	15, 25	35, 50, 75	=

Ass	Ty T ₁ stock	Horse ₄ Blue	Polyvalent (1)	12, 24, 48, 85, 100, 140	150, 300	=
779	Ty T ₁ Mouse ₄	Horse ₄ Blue	Polyvalent (1)	14, 26, 53	75	100, 200
778	Ty T ₁ 238 ser ₃	Horse ₄ Blue	Polyvalent (1)	12	25	48, 94, 115, 150, 175, 235, 350
A ₃₁	Ty T ₁ stock	Horse ₄ Blue	Polyvalent (2)	120	=	=
823	Ty T ₁ 238 ser ₃	Horse ₄ Blue	Polyvalent (2)	10	15	=
A ₃₁	Ty T ₁ stock	Horse ₄ Blue	Polyvalent (3)	90	140	=
823	Ty T ₁ 238 ser ₃	Horse ₄ Blue	Polyvalent (3)	—	15, 20	30
852	Ty 157 stock	Horse ₄ Blue	Polyvalent (3)	90	—	110, 120
846	Ty 864 stock	Horse ₄ Blue	Polyvalent (3)	40	60, 80	90
848	Ty 159 stock	Horse ₄ Blue	Polyvalent (3)	—	25, 40, 60, 75	90
911	Ty 870 phage ₃	Horse ₄ Blue	Polyvalent (3)	—	12	20, 30
A ₃₁	Ty T ₁ stock	Horse ₄ Blue	Polyvalent (12)	150	=	=
823	Ty T ₁ 238 ser ₃	Horse ₄ Blue	Polyvalent (12)	30	=	=
A ₃₁	Ty T ₁ stock	Horse ₄ Blue	Polyvalent (6)	90, 147	=	=
834	Ty T ₁ 245 ser ₃	Horse ₄ Blue	Polyvalent (6)	—	—	18
823	Ty T ₁ 238 ser ₃	Horse ₄ Blue	Polyvalent (6)	20	25	30
989	Ty T ₁ 245 ser ₃	Horse ₄ Blue	Polyvalent (6)	—	15	25, 35
911	Ty 870 phage ₃	Horse ₄ Blue	Polyvalent (6)	—	12	20, 30
989	Ty T ₁ 245 ser ₃	Horse ₄ Blue	Polyvalent (11)	15, 25	40	=
1042	C stock	S ₁	C-42	—	—	8, 14, 24, 30, 40, 50, 60 40, 50, 60, 8
985	C rough	S ₂	C-42	14, 24, 30	35	—

— = lower amounts not tested.

= = higher amounts not tested.

The horses were immunized with mixtures of vaccines and filtrates of various stock strains for a period of 8 to 12 months. Recently mixtures of vaccines and filtrates derived from various rough strains of *B. typhosus* were added to the material injected. The word "polyvalent" indicates injections of mixtures of stock strains, the number following indicates the number of injections of the rough strains.

* Each of these figures represents the number of units tested for neutralization in a group of 4 rabbits.

lizations were obtained, *i.e.*, consistent and irregular neutralizations. The irregular neutralizations which take place when the amount of reacting factors is increased beyond a certain maximum are most likely due to the fact that the mixtures contain a small amount of free reacting factors so that only the rabbits possessing a high degree of reactivity are able to respond with reactions. Quantitative comparisons of the degree of neutralizability of reacting factors derived from various filtrates can be made accurately by determining the largest amount of reacting factors *consistently* neutralized by a given serum. It does not appear safe to make quantitative estimations of the neutralizability of a filtrate by determining the largest amount of reacting factors *irregularly* neutralized by a serum since the exact mechanism of irregular neutralization is not known as yet. However, this type of neutralization offers a rough quantitative comparison of the degree of neutralizability of various filtrates by a given serum. If an analysis of this sort is made on the data obtained, the following becomes evident:

Various stock strains of *B. typhosus* show striking differences in the serum neutralizability of their reacting factors. Out of four strains chosen for this work two occupied extreme positions, one showing a high degree of neutralizability with strain homologous and strain heterologous sera (Ty T_L stock and Sera S₅ and S₆) and another strain (Ty 159 stock) showing no consistent but only irregular neutralizations with the same sera. A horse serum (Horse₅ Bleeding₁₁₄) which was prepared by injection of mixtures of various strains including Ty 159 stock also failed to give consistent neutralizations with Ty 159 stock reacting factors, although giving a high degree of irregular neutralization. The remaining two stock strains occupied intermediate positions in the degree of consistent neutralizations, one (Ty 864 stock) being of lower neutralizability than the other (Ty 157 stock). The strains preserved the same differences when tested with the polyvalent horse serum (Horse₅ Bleeding₁₁₄). The present tentative explanation of these findings is as follows:

The reacting factors of the strain showing the highest degree of neutralizability appear to be the simplest in their antigenic structure, as well as of high antigenicity. The reacting factors of the remaining stock strains appear to represent various degrees of increasing anti-

genic complexity, the Ty 159 stock strain being the most complex one. The additional complicating components, however, have to be considered of low antigenicity, as is seen from the fact that polyvalent sera, in the preparation of which there was included Ty 159 stock strain, and certain homologous monovalent rabbit sera fail to give a high degree of neutralization with these factors. Further proof of this contention is brought out by the following work with mouse strains:

The passage of *B. typhosus* stock strains through mice may or may not affect the neutralizability of their reacting factors. In one strain which was considered above as the simplest in the antigenic structure of its reacting factors (Ty T_L stock) there occurred a partial loss of neutralizability (Ty T_L Mouse₅₃ and Sera S₅ and S₆). The fact may be interpreted as an acquirement of additional components. The factors of another strain considered as the most complex ones (Ty 159 stock strain) showed no change in the neutralizability of the reacting factors after 46 passages through mice. However, there occurred an increase in the antigenicity of the factors of this strain. This fact is well illustrated by the experiments with R-309 serum homologous for Ty 159 Mouse₄ strain which shows a high degree of consistent neutralization with the reacting factors of the latter.

The passage of a strain through mice is not necessarily accompanied by an increase in the antigenicity of the factors of low neutralizability. The Ty 240 stock strain which showed factors of low neutralizability was tested with the anti-Ty T_L stock serum (S₅) as well as with an anti-mouse-passed-Ty 240-strain serum (S₆) (previous unrecorded experiments). Both sera showed low neutralizations with the reacting factors of this mouse strain. (Table II, Diagrams 1 and 2.)

As is also seen from Table II and Diagrams 1 and 2 there occurs an almost complete loss of neutralizability of reacting factors of the rough variants by the anti-stock sera. The change is illustrated by numerous experiments (Ty T_L 238 ser₂ and Ty T_L 245 ser₂, Ty 870 phage₃ vs. Sera S₅, S₆, Horses_{5/114} and _{15/115}). The loss of neutralizability is associated with acquisition of a new antigenic specificity. The "rough" reacting factors are consistently neutralized by homologous sera and by polyvalent sera, in the preparation of which there were included the rough variants (Serum S₃ and *coli* rough; Typhoid rough strains and Sera R-303 and Horses_{5/125}, _{15/127}, _{15/115}). Moreover, the two *B. typhosus* rough variants (Ty T_L 238 ser₂ and Ty T_L 245 ser₂), both derived from the same stock strain (Ty T_L), and a third *B. typhosus* rough

variant (Ty 870 phage₃) derived from another stock strain (Ty 870) varied in the degree of their neutralizability by anti-rough sera (Horses 5/114 and 15/115). The question whether the "rough" reacting factors are able to stimulate antibodies against normal reacting factors is left open.

In the work which follows an attempt was made to correlate the above findings with the morphological appearance of colonies, agglutinability and virulence of the various strains and also with the serum precipitability and lethal effect of the filtrates studied.

II. Morphological Appearance of Colonies and the Neutralizability of Reacting Factors

As is seen from Part I of this paper the stock strains of *B. typhosus* produced reacting factors of various degrees of neutralizability. The Ty TL stock strain, producing factors of high neutralizability and antigenicity and apparently the simplest in antigenic structure, was morphologically intermediately rough and was capable of prompt transformation into complete roughness. Studies on other similar strains are under way in order to determine whether this correlation can be definitely established. The reacting factors of the remaining stock strains which differed in neutralizability and were considered more complex did not display any difference in the colony morphology, all appearing normal. Neither were these strains transformed into rough variants in spite of persistent attempts. It appears, therefore, that strains of normal colony appearance differ in the neutralizability of reacting factors which they produce.

The different rough variants produced by passage through immune serum and bacteriophage appeared of morphologically identical colony structure, whilst they differed in the neutralizability of their reacting factors.

The passage of stock strains through mice which may or may not result in a change of neutralizability was not accompanied by any changes in the morphology of the colonies.

III. Serum Agglutinability of Various Stock Strains and Variants of B. typhosus and the Neutralizability of Reacting Factors

The tests were performed in the usual manner, except that 0.21 per cent NaCl solution was used for making serum dilutions for the agglu-

tionation of the rough variants. The growth of rough variants on solid media used for the tests was suspended in distilled water, washed by centrifugalization and resuspended in 0.21 per cent NaCl solution.

TABLE III
Serum Agglutination of Various Strains of B. typhosus

Strain	Serum	Degree of agglutination with various dilutions of sera										
		1-25	1-50	1-100	1-200	1-400	1-800	1-1600	1-3200	1-6400	1-12800	1-25600
Ty T _L stock	S ₅	4+	4+	4+	4+	4+	4+	4+	2+	=	=	=
Ty 157 stock	S ₅	4+	4+	4+	3+	3+	2+	1+	—	=	=	=
Ty 159 stock	S ₅	3+	3+	3+	2+	2+	2+	1+	1+	=	=	=
Ty T _L stock	S ₅	4+	4+	4+	4+	4+	4+	4+	3+	=	=	=
Ty 157 stock	S ₅	4+	4+	4+	3+	3+	3+	1+	—	=	=	=
Ty T _L stock	Horse ₅ Bl ₁₁₄	4+	4+	4+	4+	4+	4+	3+	3+	3+	1+	—
Ty 157 stock	Horse ₅ Bl ₁₁₄	4+	4+	4+	4+	3+	2+	±	—	—	—	—
Ty 864 stock	Horse ₅ Bl ₁₁₄	3+	3+	3+	3+	3+	3+	3+	3+	3+	1+	—
Ty 159 stock	Horse ₅ Bl ₁₁₄	3+	3+	2+	1+	—	—	—	—	—	—	—
Ty T _L Mouse ₃₄	S ₅	4+	4+	4+	4+	4+	4+	4+	3+	=	=	=
Ty T _L Mouse ₃₄	S ₅	4+	4+	4+	4+	4+	4+	4+	3+	=	=	=
Ty T _L Mouse ₂₆	Horse ₄ Bl ₁₀₀	4+	4+	4+	4+	4+	4+	4+	4+	3+	2+	1+
Ty T _L Mouse ₃₅	Horse ₄ Bl ₁₀₀	4+	4+	4+	4+	4+	4+	4+	4+	3+	1+	1+
Ty T _L stock	Horse ₄ Bl ₁₀₀	4+	4+	4+	4+	4+	4+	4+	4+	3+	2+	1+
Ty 159 Mouse ₃₄	S ₅	4+	4+	4+	3+	±	—	—	—	=	=	=
Ty 159 Mouse ₃₁	S ₅	4+	4+	3+	±	—	—	—	—	=	=	=
Ty T _L 238 ser ₂	S ₅	2+	—	—	—	—	—	—	—	=	=	=
Ty T _L 245 ser ₂	S ₅	2+	—	—	—	—	—	—	—	=	=	=
Ty 870 phage ₃	S ₅	2+	—	—	—	—	—	—	—	=	=	=
Ty T _L 238 ser ₂	R 303	4+	4+	4+	4+	3+	3+	2+	2+	—	=	=
Ty T _L 238 ser ₂	Horse ₅ Bl ₁₁₄	4+	4+	4+	4+	4+	4+	4+	3+	1+	—	—
Ty T _L 245 ser ₂	Horse ₁₅ Bl ₁₁₅	4+	4+	4+	3+	3+	3+	1+	±	—	—	—
Ty T _L 238 ser ₂	Horse ₁₅ Bl ₁₁₅	4+	4+	4+	4+	4+	3+	3+	2+	1+	—	—
Ty T _L 245 ser ₂	Horse ₁₅ Bl ₁₂₃	4+	4+	4+	4+	4+	4+	3+	2+	±	—	—

= not tested in higher dilutions.

The results of the agglutination tests with the various cultures, as recorded in Table III bring out the following:

There are two modes of comparison of agglutinability of various strains by immune sera: (a) the agglutination titer, namely, the highest dilution of the serum with which agglutination is obtained, and (b) the degree of agglutination in various dilutions of sera. The agglutina-

tion titer of the stock strains cannot be correlated with the degree of neutralizability of reacting factors derived from the same strains. For instance, the agglutination titer of Serum S₅ is the same for Ty T_L stock and Ty 159 stock strains, whilst there is consistent neutralization of 26 reacting units of the first and no consistent neutralization of the latter strain. In another example, the reacting factors of Ty 157 stock strain are neutralized by serum Horse₅ Bleeding₁₁₄ to a higher titer than those of strain Ty 864 stock, but the agglutination titer is higher for the second than for the first strain. On the other hand, it appears that the degree of agglutination obtained in various dilutions of sera may be an indication of differences in neutralizability of stock strains of *B. typhosus*. Thus, should one arrange the strains in order of their comparative neutralizability and in order of the intensity of agglutination in low dilutions of sera the same order is obtained for both. Thus: 1.—Ty T_L stock, 2.—Ty 157 stock, 3.—Ty 864 stock, and 4.—Ty 159 stock would fall in this order. The above described observation is to be differentiated from previously reported results showing that the agglutinating and neutralizing antibodies do not run parallel in immune sera (10).

The loss of agglutinability due to passage of strains through mice is not an indication of a change in the neutralizability of the reacting factors. For, on the one hand, there is the example of the Ty T_L mouse strains which, after passage through mice, produced reacting factors of lower neutralizability without change in agglutinability. On the other hand, in contrast to this strain, Ty 159 mouse underwent a considerable loss in agglutinability without concomitant loss of neutralizability of its reacting factors.

As is seen from the results of agglutination tests with the rough variants of *B. typhosus* and from neutralization experiments of Table II the anti-rough agglutinating and neutralizing antibodies do not run parallel. In fact, serum Horse₅ Bleeding₁₁₄ which was not able to neutralize consistently the reacting factors of strain Ty T_L 238 ser₂ has a higher agglutination titer than Serum R-203 which shows consistent neutralization of 43 reacting units of the same filtrate.

As noted before, various degrees of neutralizability exist among rough variants derived from the same stock strain. There appears to be parallelism between this and the agglutinability of the same vari-

ants by immune sera. (Horse₁₅ Bleeding₁₁₅ vs. Ty T_L 238 ser₂ and Ty T_L 245 ser₂.)

No antigen analysis (*i.e.*, somatic and flagellar antigens of Theobald Smith (22) etc.) of the various strains employed has been made as yet.*

IV. Serum Precipitability of Culture Filtrates of Various Stock Strains and Variants of B. typhosus and Neutralizability of Reacting Factors

The results of precipitation tests with various filtrates and immune sera are summarized in Table IV.

TABLE IV
Serum Precipitation of Various Bacterial Filtrates

No. of filtrate	Strain used for preparation of filtrate	No. of serum	Strain used for preparation of serum	Degree of precipitation with various dilutions of sera							
				1	1-8	1-16	1-32	1-64	1-128	1-256	1-512
A ₂₅	Ty T _L stock	S ₅	Ty T _L stock	4+	3+	±	—	—	—	—	—
852	Ty 157 stock	S ₅	Ty T _L stock	2+	2+	±	—	—	—	—	—
848	Ty 159 stock	S ₅	Ty T _L stock	4+	2+	±	—	—	—	—	—
839	Ty T _L Mouse ₃₃	S ₅	Ty T _L stock	4+	4+	1+	±	—	—	—	—
971	Ty 159 Mouse ₄₅	S ₅	Ty T _L stock	4+	4+	±	±	—	—	—	—
823	Ty T _L 238 ser ₂	S ₅	Ty T _L stock	—**	—	—	—	—	—	—	—
834	Ty T _L 245 ser ₂	S ₅	Ty T _L stock	—**	—	—	—	—	—	—	—
A ₂₅	Ty T _L stock	R 303	Ty T _L 238 ser ₂	2+	1+	—	—	—	—	—	—
823	Ty T _L 238 ser ₂	R 303	Ty T _L 238 ser ₂	4+	4+	1+	±	—	—	—	—
989	Ty T _L 245 ser ₂	R 303	Ty T _L 238 ser ₂	4+	4+	2+	1+	—	—	—	—

** No precipitation was obtained with the filtrate previously diluted 1 to 4.

A quantitative analysis of the results cannot be safely made since several dilutions of the antigens were not used in every instance. The following can, however, be concluded:

The "rough" reacting factors which are not neutralized by anti-stock sera are also devoid of precipitinogens for these sera (Ty T_L 238 ser₂ and Ty T_L 245 ser₂ strains and Serum S₅).

* The very extensive literature on inagglutinability of recently isolated *B. typhosus* strains and antigen changes occurring in the course of dissociation is reviewed by Baerthlein (16) and Hadley (17).

The appearance of neutralizing antibodies for the "rough" reacting factors is coincident with formation of anti-"rough" precipitins (Ty T_L 238 ser₂ and Ty T_L 245 ser₂ and Serum R-303).

Abundant precipitation between the filtrate and serum may occur in spite of low neutralization (Ty 159 stock and Mouse₄₆ and Serum S₆).

V. Virulence of B. typhosus Variants and Neutralizability of B. typhosus Reacting Factors

The various strains tested were transplanted into plain broth pH 7.4. 1 cc. of a broth culture previously diluted in sterile plain broth 1:4 was

TABLE V
The Virulence of Variants of B. typhosus

Total number of mice	Strain employed	Amount injected intra-peritoneally	Number of deaths after various intervals of time				Survivals
			3 hours	24 hours	48 hours	96 hours	
25	Ty T _L stock	1 cc. of 1:5 dilution	2	18	1	0	4
25	Ty T _L Mouse ₃₃	" "	0	14	3	0	8
25	Ty T _L 238 ser ₂	" "	1	4	4	0	16
25	Ty T _L 245 ser ₂	" "	0	0	1	7	18
25	Ty T _L stock	" "	0	9	1	7	8

injected intraperitoneally into each mouse. A large number of mice were tested in view of the considerable individual variations of the laboratory animals in response to the effect of the colon-typhoid group. The results are summarized in Table V. As is seen from Table V, the passage of the Ty T_L stock strain through mice did not increase its virulence. It is clear, therefore, that the change in the neutralizability of the reacting factors which occurred in the mouse strain is not related to its virulence.

There is an unquestionable lowering of virulence on the part of the rough variants of *B. typhosus*, as compared to the virulence of the stock strain from which they are derived. This was previously observed for the colon-typhoid group by Topley and Ayrton, Jordan, Orcutt, White, Goyle, Schütze and others (17). As is seen from this

paper, the lowering of virulence is coincident with a change in the neutralizability of the "rough" reacting factors by anti-stock sera.

TABLE VI
Lethal Effect of B. typhosus Culture Filtrates upon Mice

No. of mice tested	Amount of filtrate injected intraperitoneally	Strain used for preparation of filtrate	No. of filtrate	No. of deaths after various intervals of time				No. of survivals
				3 hours	24 hours	45 hours	52 hours	
25	1 cc.	Ty T _L stock	A ₂₁	3	17	3	0	2
28	1	Ty T _L Mouse ₂₃	834	9	16	2	0	1
28	1	Ty T _L 238 ser ₂	823	8	16	0	0	4
25	1	Ty T _L 245 ser ₂	834	4	8	0	3	10

TABLE VII
Lethal Effect of B. typhosus Culture Filtrates upon Rabbits

No. of filtrate	Strain used for preparation of filtrate	Intravenous dose per kilo of body weight	No. of rabbits tested	No. of deaths 1-5 hours after intravenous injection	No. of survivals
A ₁₅	Ty T _L stock	1 cc. of 1:100 dil.	4	1	3
A ₂₅	Ty T _L stock	1 cc. of 1:200 dil.	4	1	3
A ₂₅	Ty T _L stock	1 cc. of 1:400 dil.	4	1	3
911	Ty 870 phage ₁	1 cc. of 1:300 dil.	4	1	3
823	Ty T _L 238 ser ₂	1 cc. of 1:400 dil.	4	1	3
834	Ty T _L 245 ser ₂	1 cc. of 1:200 dil.	4	1	3
834	Ty T _L 245 ser ₂	1 cc. of 1:800 dil.	4	1	3
989	Ty T _L 245 ser ₂	1 cc. of 1:40 dil.	4	1	3
989	Ty T _L 245 ser ₂	1 cc. of 1:20 dil.	4	1	3
840	Ty 159 Mouse ₂₁	1 cc. of 1:800 dil.	4	1	3
840	Ty 159 Mouse ₂₁	1 cc. of 1:400 dil.	4	1	3
852	Ty 157 stock	1 cc. of 1:300 dil.	4	1	3
852	Ty 157 stock	1 cc. of 1:500 dil.	4	1	3
852	Ty 157 stock	1 cc. of 1:600 dil.	4	1	3
852	Ty 157 stock	1 cc. of 1:110 dil.	5	1	4

However, there were also differences in the neutralizability of the reacting factors derived from the two "rough" variants by anti-rough sera. No differences could be recognized in the virulence of these rough variants.

VI. The Lethal Effect of B. typhosus Culture Filtrates and Neutralizability of Reacting Factors

In these experiments mice were injected with 1 cc. of each undiluted filtrate intraperitoneally. The rabbits* received each a single intravenous injection of diluted filtrate.

As is seen from Table VI, culture filtrates of variants of *B. typhosus* as well as the stock strain from which they were derived were decidedly toxic for mice. It may also appear that the filtrate of one of the rough variants was less than the other (Ty T_L 245 ser₂ and Ty T_L 238 ser₂ respectively). However, no conclusion has been drawn as yet in view of the extreme variations in the individual susceptibility of laboratory animals to the effect of bacterial filtrates.

As is seen from Table VII, even high dilutions of filtrates irrespective of the strains and the type of variants used for their preparation proved lethal to a small percentage of rabbits. It is evident from the data brought out here that no relationships can be established between the lethal effect of *B. typhosus* filtrates and the degrees of serum neutralizability of the reacting factors present in these filtrates. There is an apparent discrepancy between the results on the lethal effect of toxic substances derived from rough cultures of the colon-typhoid group obtained by the present author and previous authors. Thus, Goyle (18) found that the filtrate of the autolyzed culture of the normal strain of *B. enteriditis* is more toxic than that of the rough strain, the tests being made in 6 mice. Schütze (19) tested washed saline suspensions of bacteria heated to 60°C. and 100°C. in mice. One mouse was inoculated with each dilution. He concluded that the rough variant of S-Aertrycke-Glasgow possessed about a quarter of the toxicity of its smooth counterpart. Heating to 100° lowered the toxicity of the S-variant by about a quarter but the toxins of the rough variant did not appear to be so labile.

The discrepancy of results may be explained by the fact that the mode of preparation of filtrates was altogether different (6) from those employed by Goyle and Schütze and also by the fact that a considerably larger number of animals was used (because of individual variations

* The rabbits used in these experiments were previously injected intradermally for titration of the phenomenon-producing potency of the filtrates.

in susceptibility of animals). However, the results of White (20) support the present author's contention. He states as follows: "Saline suspensions of smooth heat-killed bacilli excel those of rough bacilli in toxicity. However, alcohol-treated rough *B. typhosus* and *Salmonella* bacilli exhibit for rats and rabbits a toxicity which hardly falls short of that of smooth. It seems probable that the potential toxicity of the rough derivative approximates to that of the smooth parent organism and that contrasts in actual toxicity are due to differences in the rate of liberation within the animal body or into fluid culture medium."

DISCUSSION

From previous studies it has been concluded that the phenomenon described is the result of a state of induced susceptibility to antigenically specific and neutralizable toxins from a great variety of microorganisms produced in tissues spontaneously resistant. Inasmuch as this phenomenon can be elicited in various organs and inasmuch as it is indicative of severe injury produced by specific bacterial toxic substances, it possibly expresses in "acute" form the underlying mechanism in the pathogenesis of spontaneous infectious diseases. In the light of this conception various problems of both practical and theoretical interest present themselves:

In previous studies the injury producing factors (*i.e.*, reacting factors) of the phenomenon under discussion were shown to be species specific. The work embodied in this paper demonstrates also strain and variant specificity.

By the "strain specificity" is meant the fact that various strains, chosen at random, of a given species of bacteria produce reacting factors which differ in their neutralizability. The differences are explainable on the basis of various degrees of antigenic complexity.

The "variant specificity" is demonstrated by a number of facts.

The passage through mice induces in certain strains a partial loss of neutralizability of reacting factors. This is probably due to variation towards smoothness with an increase in the antigenic complexity. As a matter of fact, the reacting factors of a strain considered as the simplest in their structure underwent a partial loss of neutralizability after passages through mice, whilst the neutralizability of reacting

factors of a strain found to be the most complex before the passage through mice was not influenced by the latter procedure. The other type of "variant" specificity is conditioned by the change of a strain into rough. The "rough" reacting factors acquire a new specificity.

The above described changes in the neutralizability of the reacting factors taken together cannot be consistently correlated with colony morphology, serum agglutinability and virulence of strains, nor with the serum precipitability and general toxicity of the culture filtrates. This subject has been taken up in detail in the text.

Inasmuch as the occurrence of bacterial variation *in vivo* is a recognized fact, the relationship established in this paper between the neutralizability of toxic substances and bacterial variation offers an opportunity to add new data to our immunological understanding of the course of typhoid fever and possibly other infectious diseases.

As seen previously, *B. typhosus* strains differ in their ability to undergo variation. If a given infection is produced by a "normal" strain incapable of changing into a "rough" one the toxic substances (*i.e.* reacting factors) will be neutralized by the "normal" antibodies developing in the course of the disease. However, should the strain be capable of prompt change into "rough" the production of antibodies will promote the variation and there will appear in the body "rough" reacting factors non-neutralizable by the "normal" antibodies. Until now, the toxicity and change in specificity of "rough" bacterial filtrates have not been recognized. It was known, however, that once a strain becomes rough it loses its virulence and, therefore, the promotion of variation might be considered as a helpful process. The work embodied in the present paper demonstrates the injury producing effect of the "rough" bacterial substances both in general toxicity and in their effect upon prepared skin sites. It also demonstrates the change in their antigenic specificity. In the light of these facts it may be assumed that the course of a spontaneous infectious disease would also depend on the ability of a given strain to undergo variation, the disease being more protracted when such a change occurs, and lasting until the development of the anti-rough antibodies.

Inasmuch as the reacting factors of normal strains also vary in their neutralizability and the degree of their antigenicity, it might be ex-

pected that the course of the disease would also depend on these immunological properties of the invading organisms.*

It is probable that the above considerations will prove of importance in planning the serum therapy of infectious diseases. Assuming that they are so, it would seem necessary to prepare immune sera sufficiently potent to neutralize the "normal" reacting factors even of low neutralizability. For this purpose advantage could be taken of the above described increase in antigenicity obtained by means of mouse passage. The serum should also contain antibodies specific for "rough" reacting factors since the presence of such factors may be expected *a priori* in any disease running a protracted course and since the injection of anti-normal antibodies will promote their appearance, provided the invading organism is capable of the transformation. The author is attempting to prepare in this manner antityphoid and anti-*coli* immune sera for ultimate therapeutic use.

CONCLUSIONS AND SUMMARY

In this paper there is reported the effect of bacterial variation upon the reacting factors of the phenomenon of local skin reactivity to bacterial culture filtrates. By means of the method described in the text the following results were obtained:

Various stock strains of *B. typhosus* were found to produce reacting factors which differed in their neutralizability by anti-stock immune sera. The reacting factors of low neutralizability also possessed low antigenicity. The passage of strains through mice brought about in one instance a lowering of neutralizability of reacting factors and in another instance had no effect. It was possible to increase the antigenicity of the reacting factors by passage of the strain through mice.

* It is of interest to mention here the work by Durham (21), who many years ago assumed that the original typhoid infection is produced by various strains of allied bacilli. According to his view, if the different strains are similar, antibodies are produced to the several strains and no relapses occur. If a particular strain predominates in the primary attack, though effective antibodies to this strain may be produced and may bring the attack to an end, few or perhaps no antibodies will be produced to the less abundant strains. These strains may then be responsible for a relapse.

Transformation of "stock" strain into "rough" brought about the formation of reacting factors of new specificity. The "rough" reacting factors were neutralized by homologous sera and also differed in their neutralizability.

Reacting factors of various degrees of neutralizability were produced by stock strains which did not display any difference in colony appearance (with one exception). The "rough" reacting factors appeared in strains of typical "rough" colony morphology. However, "rough" strains of the same degree of roughness morphologically differed in the neutralizability of their reacting factors. The changes in neutralizability elicited by passage through mice had no influence on colony morphology.

There seemed to be a parallelism between the degree of agglutinability of stock strains by anti-stock sera and the degree of neutralizability of reacting factors of the same strain.

It was possible to obtain a partial loss of neutralizability by passage through mice with and without change in agglutinability. The agglutination titer of anti-rough serum was not an indication of its anti-rough neutralizing properties.

The degree of neutralizability of "rough" reacting factors paralleled the specific serum agglutinability of the strains producing them.

Abundant precipitation occurred in mixtures of toxic filtrates and immune serum in spite of low neutralization. The filtrates of rough cultures non-neutralizable by anti-stock sera failed to precipitate with the latter. The appearance of neutralizing anti-rough antibodies was coincident with the development of anti-rough precipitates.

The rough strains possessed a lower virulence than the stock strains from which they were derived. However, there was no difference in the virulence of "rough" strains, the reacting factors of which differed in their neutralizability. The filtrates containing "rough" reacting factors were seemingly as lethal to mice and rabbits as were filtrates of stock cultures of *B. typhosus*.

The possible relation of these findings to the immunological understanding of the course of infectious diseases and to serum therapy is discussed.

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STUDIES ON TUBERCULOSIS

By

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AND CLAUDE E. FORKNER, M.D.



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(From the Laboratories of The Rockefeller Institute for Medical Research)

PLATES 1 TO 10

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CHAPTER I

REACTION OF THE CONNECTIVE TISSUES OF THE NORMAL RABBIT TO LIPOIDS FROM THE TUBERCLE BACILLUS, STRAIN H-37

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PLATES 1 TO 7

INTRODUCTION

This series of studies represents an effort to analyze how far each one of the manifestations of tuberculosis can be related to specific chemical substances isolated from the tubercle bacillus. The factor considered here is the production of new tissue, with the formation of the tubercle as the most significant element. We shall show that the substances from the tubercle bacillus which give typical tubercular tissue are limited to the lipid fractions. Epithelioid cells are not produced in the normal animal by the soluble tuberculo-proteins or by the polysaccharide derivatives. The fractions used were from the human strain of tubercle bacilli, H-37; but comparative studies are being made with fractions from the bovine and avian strains as well as from the timothy grass bacillus (1). These studies with the lipoids have been made for the most part in the normal rabbit.

During the years since 1891, when Koch (2) first reported his observations on the effect of injecting killed tubercle bacilli, many studies have been made of the changes produced in tissues by dead tubercle bacilli and by lipoids extracted from them; the reactions produced by other lipoids extracted from various bacilli and from certain of the organs of the body, such as the liver, have also been described. This literature was analyzed in our first report on the biological reactions to fractions from the tubercle bacillus (Sabin and Doan, 3). Weyl (4) was the first to use chemical extracts from the tubercle bacillus in such work. As a result of many studies, the effect of any lipid injected into the tissues has come to be considered as belonging to the common type of the so-called foreign body reaction (Morse and Stott, 5; Ray and Shipman, 6). The individual differences in results

are difficult to evaluate both on account of an inadequate chemical separation of the different types of lipoids and because of obscurities in the analysis of the complex effects in the tissues. In general they indicate that the tubercle, the lesion which, in association with the tubercle bacillus, is characteristic of the disease, can be produced either by dead tubercle bacilli or by lipoids extracted from them.

The chemical fractions have been made available through a plan for cooperative research organized by the Research Committee of the National Tuberculosis Association (William Charles White, Bureau of Hygiene, Washington, D. C., Chairman). Acid-fast bacilli of standard strains have been grown in large quantity under uniform conditions, on Long's synthetic medium, by H. K. Mulford Company and by Parke, Davis and Company. A complete bibliography of all the work under this Committee has been published recently by the National Tuberculosis Association (1). Chemical analyses of bacilli and filtrate have been made by Dr. Treat B. Johnson and Dr. R. J. Anderson and their associates at the Sterling Chemistry Laboratory of Yale University, and of the filtrate by Dr. E. R. Long and Dr. F. B. Seibert, Department of Pathology, University of Chicago. So comprehensive has been the plan of this work that chemical products of uniform standard, certainly products that can be used for comparable biological studies, are now becoming available in considerable quantities from each of the several members of the acid-fast group of bacteria.

In these papers we shall use the terms "tuberculous" and "tubercular" in the senses recommended by the National Tuberculosis Association. "Tuberculous" tissue and tubercle will be understood to mean lesions produced in the actual disease, and hence under the influence of the tubercle bacillus; while "tubercular" tissue and tubercle-like structures will be used to describe lesions resembling those of the disease, but produced by the chemical products from the bacillus. The need for the term "tubercular" is an indication that lesions resembling tubercles—more specifically, diffuse and massive collections of epithelioid cells—occur in other pathological conditions than those set up by the tubercle bacillus. In this paper we shall refer to lesions produced by chemical fractions from the tubercle bacillus, involving epithelioid cells and giant cells of the Langhans type, as characteristic "tubercular" lesions whether the epithelioid cells are diffusely scattered in a tubercular granulation tissue, or are in the form of compact clumps or tubercle-like structures. All the other connective tissue reactions found in the disease and reproduced by the chemical fractions, such as the draining of leucocytes from blood to tissues, increase in cell division or in phagocytic activity of clasmatocytes, foreign body giant cells, increase in undifferentiated fixed connective tissue cells, or in fibroblasts, new growth of blood vessels, increase in lymphocytes either from the blood or from a local development in the tissues, will be spoken of as general or non-specific lesions.

During recent years, the development of methods for studying living cells by the supravital technique and by tissue culture has given new

criteria for estimating and differentiating biological reactions. These studies have made it possible to detect epithelioid cells, the essential cellular units of the tubercle, in fresh tissues, even when they are few and scattered, with more certainty than in sections. In a study of giant cells with the supravital technique, reported in this monograph (7), evidence is cited to show that the so-called Langhans giant cell is a direct derivative of a single epithelioid cell, whereas the foreign body giant cell (8) results from the fusion of monocytes, epithelioid cells, and possibly other mononuclear cells. The name epithelioid giant cell has therefore been suggested for the former type to indicate that it is a multinuclear epithelioid cell. It has been shown that in the disease tuberculosis, and around foreign bodies as well, both types of giant cells occur, but in varying proportions according to the type of stimulus. As is well known, the epithelioid giant cell predominates in tuberculosis.

It is feasible therefore to stress the production of epithelioid cells and epithelioid giant cells, in contrast to other mononuclear and multinuclear forms, as perhaps the most significant factor in the pathology of tuberculosis.

REACTION TO THE PHOSPHATIDE A-3

In the analysis of the lipoids made by Anderson, a phosphatide designated by him A-3 has proved to have the most significant biological properties. It not only produces tubercular tissue in greatest amount, but it may act as an antigen; in the one instance its effect is to increase the lesions of the disease, in the other it may tend to reduce them. The specific cellular stimulant in the phosphatide is a saturated fatty acid.

From Table I it will be seen that we have received from Dr. Anderson¹ (9) four lipid partitions, a phosphatide A-3, a so-called purified wax, a soft wax, and glycerides. Their respective solubilities are shown in the table. The total lipoids Dr. Anderson reports as about 24 per cent of the dry bacilli, Strain H-37. The waxes comprise 46 per cent (11 per cent of the dry bacilli); and the phosphatide 20 per cent (5

¹ Beside the references of papers already published, Dr. Anderson is now writing a complete account of his chemical analyses up to the present time.

TABLE I
Effects of Tuberculo-Lipoids Prepared by Anderson Compared with Effects of Tubercle Bacilli

Living Tubercle Bacilli

1. Leucocytes, neutrophilic (acute, 24 hours).
2. Clasmatocytes (phagocytizing leucocytes).
3. TUBERCLES (monocytes, epithelioids, giant cells).
4. Lymphocytes (chronic).

Dead Tubercle Bacilli

1. Leucocytes, neutrophilic (acute, 24 hours).
2. Clasmatocytes (phagocytizing leucocytes).
3. TUBERCLES (monocytes, epithelioids, giant cells).
4. Lymphocytes (chronic).

Lacks power of extension of lesions.

Effects of Intra peritoneal Injections of Chemical Fractions from Tubercle Bacilli, Strain H-37

Tuberculo-proteins	Lipoids					Polysaccharides
	Acetone-insoluble			Acetone-soluble		
	Alcohol-ether-soluble	Chloroform-soluble		Alcohol-ether-soluble		
		<i>Purified wax</i> 1. Leucocytes, neutrophilic 2. Clasmatoctyes 3. Tubercle-like structures ++ 4. Increased connective tissue	<i>Soft wax</i> 1. Leucocytes, neutrophilic 2. Clasmatoctyes 3. Tubercle-like structures +++	<i>Glycerides</i> Not tested		
Leucocytes, neutrophilic Clasmatoctyes phagocytizing leucocytes	<i>Phosphatide A-3</i> 1. Leucocytes, neutrophilic 2. Clasmatoctyes 3. Tubercular tissue ++++ 4. Lymphocytes					Leucocytes, neutrophilic Clasmatoctyes phagocytizing leucocytes

Phosphatide A-3	Purified was	Soft was	Glycerides	
A. Oleic acid B. Palmitic acid C. Glycophosphoric acid (all non-specific connective tissue) D. Glucose E. Sugar acid	A. Unsaponifiable substance Increased connective tissue	A. Fatty acid III 1. Leucocytes 2. Clasmato-cytes 3. Tubercle-like structures + + + +	A. Fatty acid IV Few epithelioid cells	B. Fatty acid V Epithelioid cells +
F. Fatty acid I 1. Leucocytes 2. Clasmato-cytes 3. Tubercle-like structures + + + + + 4. Lymphocytes	B. Fatty acid II 1. Leucocytes 2. Clasmato-cytes 3. Tubercle-like structures + + 4. Increased connective tissue		<i>Separated by fractional distillation</i> Tuberculo-stearic acid Optically inactive Epithelioid cells + Increased connective tissue	

per cent of the dry bacilli). In the lower half of the table are listed the chemical products derived from the four major fractions, so that it is possible to compare the biological activity of the larger molecules of the original fractions with the active principle for the formation of epithelioid cells.

Of all the lipoids tested, the phosphatide A-3 and the saturated fatty acid derived from it are the most potent in producing tubercular tissue. The other lipid fractions, purified wax, soft wax, and glycerides, contain active fatty acids to a less degree.

In any study of the action of the lipid fractions with respect to their power to produce lesions, the route of injection is important. At the present time we shall discuss the effects of the intraperitoneal route only; the effects of intravenous (10) and subcutaneous injections, also significant, require separate presentation.

The lipoids produce a marked new growth of general connective tissue as well as tubercular granulation tissue and tubercle-like structures; only the development of epithelioid cells is stressed in the table, and is designated by the words "tubercular tissue," or "epithelioid cells," or "giant cells" (the Langhans or rosette giant cells, derived from the epithelioid cell).

The criteria which have been met for the designation of any reaction as "specific," or positive, are as follows:

1. The maturation and multiplication of new monocytes. The epithelioid cell is not the immediate reaction to active chemical substances. Preceding its appearance there is a maturation of new young monocytes, identified with the supravital technique by their characteristic reaction to neutral red and demonstrated in sections in division. An active cell division is an important feature, since, though monocytes occur normally in considerable numbers in the omentum of certain rabbits, the specific lesions produced involve an increase in these cells.

2. The occurrence of epithelioid cells by the 4th day.

3. The presence of epithelioid cells, after from seven to twelve daily doses, readily demonstrable, in the supravital technique, as the predominating reaction, and giving in fixed sections structures which closely simulate the tubercles of the disease.

The criteria for the identification of the living monocytes have been given in a previous publication (11). The essential features are the rosette of small bodies that react to neutral red with a uniform salmon tint and the masses of tiny mitochondria around the rosette and excentric nucleus. The characteristics of the living epithelioid cell stained with neutral red have been described by Cunningham

et al. (12). In our first report on the action of the chemical fractions (3), an epithelioid cell from the blood stream of a rabbit with tuberculosis (Fig. 3) was shown on the same plate with a similar epithelioid cell (Fig. 5) produced chemically by the phospholipin. In the epithelioid cell the rosette is large owing to the enormous numbers of tiny bodies reacting to neutral red as do the fewer bodies of the normal monocyte.

There is some difference of opinion concerning the relationships of the cells of the connective tissues; one group of investigators regard all mononuclear forms as different physiological states of the same strain of cells; another group hold the opinion that they represent different functional strains. The evidence at hand seems to us to give weight to the view that monocytes, epithelioid cells, and the rosette giant cells derived from epithelioid cells, represent a strain of connective tissue cells somewhat different from the macrophages or clasmatocytes. Whether this view be correct or not, the tissue reactions recorded as positive in these experiments embrace a major response of cells answering to the above criteria for monocyte, epithelioid cell, and Langhans' giant cell.

Our first report (3) included the preliminary biological testing of the phosphatide, designated A-3, from the human strain of tubercle bacilli H-37. Four rabbits (R 153, R 158, R 159, and R 160) were given twelve intraperitoneal doses of the phosphatide in amounts averaging 80 mg. per dose, in 10 cc. distilled water. The phosphatide produced massive tubercular tissue in the omentum, under the serosal coat of the intestine, in the mesentery, and under the parietal peritoneal lining. The reaction was predominately one of epithelioid cells and Langhans' giant cells, either diffusely scattered or aggregated into clumps simulating tubercles, as illustrated in Figs. 10, 11, and 12 in an earlier report (3). The animals showed no toxic symptoms; there was no fever and no loss in weight. There was a definite rise in monocytes in the blood, with some qualitative changes suggestive of the transition from monocyte to epithelioid cell, not, however, as marked as in the disease itself. There was in each case a slight fall in red cells and in hemoglobin.

These observations have since been confirmed and extended in a large series of animals.

In the present study, twenty-one normal rabbits (Table II) received the phosphatide A-3 intraperitoneally, uncomplicated by other procedures. Thus, with the four of the preceding report, twenty-five animals have been included in the work. The dose which has been generally used, 80 mg., represents the amount of the phosphatide in 1.6 gm. of bacilli, this substance amounting to 5 per cent by

weight of the dry bacilli. When the phosphatide was first isolated in 1927, it was possible to get a fine dispersion of the particles by shaking the substance in water in a test-tube. Two years after the original isolation, it was found necessary to grind it in a mortar while adding water drop by drop. In this manner a uniform suspension could be obtained in which no flocculi could be seen with a hand lens and from which none settled out on standing.

The original finding of massive tubercular lesions and the fact that the phosphatide is non-toxic *via* the intraperitoneal route have been confirmed. Out of the group of twenty-five rabbits, four became ill, probably independently of the injections, except in so far as there was accidental secondary infection. Two (R 462 and R 321) developed diarrhea; one (R 462) died, but the cause of death could not be determined at autopsy; there was no peritonitis or pneumonia. The other (R 321) was killed and showed no peritonitis, but in one lung there was a patch of atelectasis in which the bronchi were filled with leucocytes and the lymphatics with a coagulum. A third animal (R 463) died of pneumonia, and a fourth (R 376) died of undetermined cause. To the group of twenty-five rabbits, fifteen more may be added, in connection with observations as to the non-toxic nature of the phosphatide. Each of them received twelve intraperitoneal injections of 80 mg. of the phospholipin and was then inoculated with living tubercle bacilli. None of these fifteen animals showed any symptoms from the phosphatide injections.

In the studies with the phosphatide there have been no complications from the presence of a diluting menstruum; the sterile distilled water used was itself inert to the connective tissue cells. With the four exceptions just mentioned, there has been no infection. The phosphatide itself could not be sterilized, but other precautions for sterility were taken, and when there were signs of infection carried in from the skin or from puncture of the intestine by the needle, cultures were taken. Routine cultures were not made. A smooth and glistening serosal lining was taken as evidence of no peritoneal infection.

Twenty-four of the forty animals which received intraperitoneal injections of the phosphatide had complete studies of the blood cells. There was a tendency to an irregular rise of monocytes in the peripheral blood, averaging 20 per cent of the total white blood cells at the highest point, with a range of from 12 to 40 per cent, and total numbers of from 1,250 to 7,500 monocytes per c.mm.

Fifteen of the rabbits showed an average fall in the total red blood cell count of 850,000 per c.mm., and a decrease of 10 per cent in the hemoglobin during the injections (see (3), Charts 4 and 5). To ascertain if this slight anemia should be attributed to the phosphatide

tide *per se* or to other factors involved in the experiments, four controls were made.

Rabbit R 709 was given twelve daily intraperitoneal doses of 10 cc. of distilled water; R 730, twelve doses each of 10 cc. normal salt solution; R 664 twelve doses each of 10 cc. normal saline plus 80 mg. of the phosphatide; and R 1016 one dose of 1 gm. of phosphatide in 20 cc. distilled water. The rabbit receiving repeated injections of distilled water (120 cc. total) did not show anemia at any time, whereas the other three animals each developed a definite anemia in the course of the

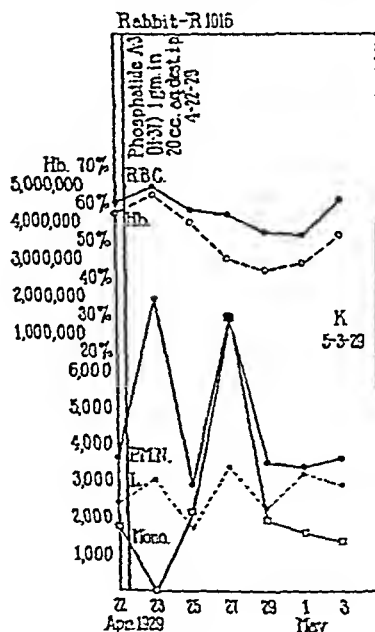


CHART 1

experiment. Chart 1 records the blood changes observed in Rabbit R 1016 over a period of 11 days, following one injection of 1 gm. of phosphatide intraperitoneally in 20 cc. distilled water, an amount of phosphatide equivalent to that usually injected in divided doses over the same period. Not only was there a definite, though transient decrease in red blood cells, but the circulating monocytes, on the 5th day after the injection, reached 7,500.

From these experiments and others in which the intravenous route was employed, it is evident that the phosphatide itself has a tendency to produce an anemia; it is also clear that, as the monocytes are in-

TABLE II

Protocols

The Effect of Intraperitoneal Injections of Phosphatide A-3 from Tubercle Bacilli, H-37, Given in Distilled Water, in Producing Tubercular Tissue in 21 Rabbits

No. of rabbit*	Dose	Results
R 819	1 dose of 80 mg. in 10 cc. aq. dest.	Milk spots of omentum accentuated by phagocytosis of lipid in large round cells. Marked emigration of leucocytes and dilatation of vessels
R 574 R 820	2 doses of 80 mg.	Milk spots increased in size and number by phagocytic cells. Free leucocytes fewer; many engulfed in clasmatocytes
R 575 R 821	3 doses of 80 mg.	Milk spots more massive and more numerous. Phagocytic cells predominate; leucocytes few and mostly in clasmatocytes. Monocytes and epithelioid cells in active division
R 478	7 doses of 10 mg.	Omentum contains many small groups of epithelioid cells; many dividing
R 482	15 doses of 20 mg.	Omentum, slight general thickening with epithelioid cells
R 462	5 doses of 80 mg.	Developed diarrhea; found dead, cause not determined; no peritonitis. Omentum showed increase in young basophilic mononuclear cells and epithelioids. No tubercle-like structures. No giant cells
R 463	6 doses of 80 mg.	Died of pneumonia. Omentum considerably thickened with diffuse epithelioid cells. Tissues infiltrated with leucocytes. Many abscesses
R 664	11 doses of 80 mg.	Thin central part of omentum showed milk spots of epithelioid cells; rest extensively thickened with tubercular tissue of great variety. Epithelioid cells and epithelioid giant cells
R 319 R 321 R 376 R 380 R 381 R 382 R 383	11 to 14 doses of 80 mg.	Omenta massively thickened with tubercular tissue. Peritoneum extensively involved. Omenta of R 376, R 380, and R 381 used by Dr. Anderson for recovery of a phosphatide by chemical analysis. Omenta of R 319 and R 382 used by Drs. Richardson, Lobell, and Schorr for respiratory tests. R 321 developed diarrhea and was killed; tissues showed marked necrosis. R 376 died, cause undetermined

* These are serial numbers of the work of the department covering a term of years.

TABLE II—*Concluded*

No. of rabbit	Dose	Results
R 1016	1 dose of 1 gm. in 20 cc. aq. dest.	Central area of omentum was thin but had large milk spots of epithelioid cells. Rest of omentum massively thickened with tubercular tissue; typical epithelioid cells and epithelioid giant cells with fine neutral red bodies predominating
R 368	13 doses of 80 mg.	Killed 1 mo. later. Excellent state of nutrition; gain in weight from 2,300 to 2,750; much yellow adipose tissue; adhesions opposite place of injections. Omentum adherent; considerably thickened with scattered epithelioid cells and small tubercle-like masses. Richly vascularized
R 377	" "	Killed 3 mos. later. Excellent condition; gain in weight 1,800 to 2,475. Omentum much less thickened but had more adipose tissue than usual. No scattered epithelioids but several compact nodules with a few epithelioid giant cells
R 379	" "	Killed 5 mos. later; gain in weight from 1,840 to 2,550. Adhesions opposite place of injection. Omentum moderately thickened with fibrous tissue. Only two nodules of epithelioid cells large enough to see in the gross. No diffuse epithelioid cells but a few small clumps

creased in the tissues, irregular showers may appear in the peripheral blood. Further analyses of the effects of the intravenous phosphatide on the bone marrow are being made and will be reported later.

The injection of living or dead tubercle bacilli is followed in the first 24 hours by an emigration of leucocytes from the vessels into the tissues and their subsequent phagocytosis by clasmatoocytes. Within 4 days epithelioid cells appear, followed by the development of tubercles (Kageyama, 13, and Lewis and Sanderson, 14). The same acute response, an emigration of leucocytes and their destruction by clasmatoocytes, follows the intraperitoneal injection of every substance isolated from the bacillus. The different substances vary however considerably as to whether repeated injections cause a fresh emigration of the leucocytes or not (Table II).

Of all the lipoidal substances it is the phosphatide, not the so-called waxes, that produces the most massive tubercular tissue. The reac-

tion to the injection of the phosphatide A-3 following one, two, and three doses is, however, complex and does not consist in the immediate formation of epithelioid cells either diffuse or in tubercles. After from seven to fourteen doses the formation of massive tubercular tissue is constantly found.

24 hours after the first dose (Rabbit R 819), the most striking change in the peritoneal cavity was in the omentum and consisted in the accentuation of the milk spots (protocols in Table II). This was due in part to the emigration of large numbers of neutrophilic leucocytes into the milk spots, but more to the fact that all the cells of the milk spots had rounded up and became highly phagocytic both to the lipoid and to leucocytes. Since the milk spots of the normal omentum in the rabbit are made up of monocytes, young clasmatoocytes, and still more primitive cells, all of these types must be involved in the original phagocytosis of the phosphatide.

After the second dose (Rabbits R 820 and R 574), the milk spots were denser with phagocytic cells, but the free leucocytes were less in number because those called out early had been largely phagocytized and the second injections did not produce as much irritation as the first. Following the third dose (Rabbits R 821 and R 575), only a few leucocytes remained either free or in clasmatoocytes, and the further injections of the phosphatide did not call out more leucocytes from the vessels.

At first the process was limited to the original milk spots; but by the third dose new milk spots were forming. In the supravital preparations the dense milk spots were made up of large round cells of nearly uniform type heavily loaded with large vacuoles which stained a uniform red color in neutral red. In fixed sections these cells appear highly vacuolated (Plate 1, Figs. 1-3, Rabbit R 821), the lipoidal substances having dissolved out in the dehydration and embedding. Stained for fat, these cells were negative to Sudan III; with Scharlach R most of the material was negative, but along the edges of the large, unstained globules were great numbers of tiny droplets which stained with the characteristic reaction of a fat. The phospholipin itself did not stain either with Sudan III or with the Scharlach R, so that if this intracellular material which gives the fat reaction came from the phosphatide, it must have been the product of some change within the cells.

On Plate 1, Fig. 1, one of the vacuolated cells (upper left corner) contains a leucocyte partly digested, and in Fig. 2 there are two such cells, each containing a leucocyte with the nucleus still recognizable, and a third, near the center, showing only débris. This reaction after three doses is minimal, and it will be noted that there is only one free leucocyte (Fig. 2) in the three figures.

The milk spots as studied in the fresh omentum and in sections had a few smaller cells, well shown in all three figures, and identified as monocytes and epithelioid cells. Two cells are clearly shown along the left border in Fig. 1, one of them with

the dense cytoplasm and excentric nucleus characteristic of a small epithelioid cell. A small group of young mononuclear cells is in the center of Fig. 3, two of them in division. This material showed much cell division, most frequently in the typical monocytes; the highly vacuolated cells however showed an occasional nuclear figure, while the endothelium of the small vessels and the adventitial cells were dividing freely. In this tissue on the 4th day after the first dose, the vacuolated cells predominated, while monocytes and typical epithelioid cells were in minimal numbers; but later the reverse was the case, the maturation of new monocytes and their transformation into epithelioid cells becoming the dominant reaction.

During the 2nd week of the reaction in the omentum to repeated injections of lipoids, the adipose tissue became gradually replaced by the new tubercular tissue and during this process fat cells appeared vacuolated in sections, owing to the breaking up of the fat into smaller globules. In the shrinking fat cells the droplets readily reacted to Sudan III, and thus these cells could be discriminated from the cells of the early reaction to the phosphatide in which most of the material was negative to fat stains.

All the animals in Table II which received from seven to twelve doses of the phosphatide exhibited a formation of tubercular tissue, the amount of which was in a general way proportional to the dose.

Rabbit R 478 had seven daily intraperitoneal doses of 10 mg. of the phospholipin in 2 cc. of distilled water and the omentum was studied 24 hours after the last dose. In this tissue, small clumps of epithelioid cells were found (Plate 2, Fig. 5). The total amount of the phosphatide given, 70 mg., was less than the average single dose, 80 mg. In this rabbit the omentum had accentuated milk spots but there was not enough of the new tissue to cause in the interspaces any change detectable with the unaided eye; nor was there any replacement of the fat by the specific tubercular tissue, that is, there was no thickening of the membrane. This condition in which only the milk spots are involved represents the first stage in the reaction in the omentum. Supravitality the milk spots themselves consisted predominantly of epithelioid cells. Some of them had two nuclei, but no larger giant cells were seen. The sections also show that the milk spots are clumps of epithelioids; in Fig. 5 is a clump of about twenty typical epithelioid cells, one of which is in division. The cells show the dense cytoplasm and the excentric nuclei characteristic of epithelioid cells of the actual infection. Note the absence of leucocytes both in this figure, and in Fig. 6.

In Fig. 6 is shown a similar small tubercle-like structure taken from the wall of the cecum just under the serosal coat in a rabbit (R 319) which had twelve intraperitoneal doses of 80 mg. of the phosphatide. It will be observed that these epithelioid cells have a much less dense cytoplasm than those of Fig. 5; in fact, except for a central core the entire cytoplasm appears foamy. In the supravital

technique, these cells had larger bodies reacting to neutral red than those in the typical epithelioid cell. This difference in size of neutral red bodies can be seen in the photographs from living cells from Rabbit R 664 (Plate 1, Fig. 4, and Plate 3, Figs. 7 and 8). This rabbit received eleven doses of 80 mg. of a phosphatide sample 2 years old. The reaction was typical but not as massive as with the animals experimented on earlier with the same dosage. In the omentum of this animal (R 664) there was in the gross a marked accentuation of the milk spots, shown in Fig. 4, but not enough change in the interspaces to be detected with the unaided eye. Fig. 4 is not from a section but from a spread of the omentum supravitaly stained with neutral red and photographed while the cells were still living. The milk spots showed an almost uniform reaction; the cells in the center of the photograph are paler, merely because they were beginning to fade. This is perhaps as pure a reaction toward one type of cell as can be obtained in the tissues of the animal; it is, so to speak, a culture of epithelioid cells in the omentum. Fig. 7 shows the edge of one of the milk spots in the same omentum at a higher magnification; there is one giant cell of the rosette type. This giant cell and the epithelioid cells near it show the bodies which react to neutral red, and photograph dark, as larger and more irregular in size than the neutral red bodies in the two cells of Fig. 8, taken from an interspace of the same omentum. In the latter figure the bodies are finer and more densely packed. In this animal the vast majority of the cells were of the type with the coarser neutral red bodies, and in fixed sections the majority of the epithelioid cells had a foamy cytoplasm intermediate in type between the cells of Fig. 5 and Fig. 6; the cytoplasm was dense with a high dry lens but appeared foamy with oil immersion lenses. The sections of this material (Rabbit R 664) showed much tubercular granulation tissue, many epithelioid giant cells, and many tubercle-like structures.

To test whether time was a factor in regard to these two types of epithelioid cells, the one with the foamy cytoplasm and the other with the dense cytoplasm, a rabbit, R 1016, was given in one dose the total amount of the phosphatide usually given in twelve divided doses, namely, 1 gm. in 20 cc. distilled water intraperitoneally. It was killed 12 days later. The changes in the blood have already been referred to (Chart 1). The middle portion of the omentum showed large milk spots with no thickening of the interspaces; and the rest of the omentum was dense with nodules of pink tissue markedly resistant on cutting. There was also some of the specific tissue, that is, some epithelioid cells, in the thoracic lymph nodes, along the internal mammary veins, and in the ventral pericardium. Supravitaly the clumps of new cells in the omentum were found to be predominately epithelioid cells with typical fine neutral red bodies, but here and there was one with the coarse granules corresponding to an occasional epithelioid cell with foamy cytoplasm (like the cells of Fig. 6 in the sections). This was the same material that gave a predominance of the epithelioid cells with coarse granules when given in divided doses. There were also many typical epithelioid giant cells. The experiment suggests that the epithelioid cells with the coarse granules

the cells with the fine neutral red bodies if sufficient time is allowed; or if the cell contains some substance which it has phagocytized, it has the power of ingesting this substance into finer and finer particles.

The second stage of the extreme reaction from twelve doses of 80 mg. of the phosphatide given intraperitoneally, which was described in the original report upon four rabbits, has been studied in seven rabbits.

The tissues from these animals were examined supravivally to confirm the presence of a massive development of the tubercular reaction and then the omenta were used either for the chemical recovery of phospholipin by Dr. R. J. Anderson, or the tissues were used for histological study by Dr. H. B. Richardson, Dr. R. O. Lobell, and Dr. E. Schorr of Cornell University, New York, for studies on the respiratory rate of the animals.

At this dosage of 80 mg., the omentum became massive with tubercular reaction (Figs. 9-13) within 12 to 14 days, being transformed into a dense membrane with many irregular nodules at least 2 to 3 cm. thick. The reaction was not one of acute infection; it was not a peritonitis of bacterial origin; there was no tendency to adhesions; except when there was an accidental bacterial infection, there was no infiltration with leucocytes after the repeated dosages except in relation to the nodules of caseation; the serosal lining was intact, and beneath the serosa were extensive masses of the new tissue. In this massive reaction not only the thin part of the omentum showing milk spots but the denser normally filled with fat cells became involved; these cells disappeared almost completely and their place was taken by the tubercular masses. Beside the effect on the omentum, there was a widespread subserosal reaction in the peritoneal cavity, shown by extensive tubercular tissue in the mesentery, the outer coat of the stomach, the capsule of the spleen, the mesentery between liver and stomach, the capsule of the liver, the diaphragm, the parietal peritoneum, and the connective tissue around the bladder and the reproductive organs. These lesions were scattered over an extensive area that it was not possible to estimate the actual amount of new tissue formed in the 2 weeks of the experiment, but judging from the omentum the entire mass was probably comparable in size to a hen's egg, a rate of growth of the new tissue resembling that of a rapidly growing neoplasm.

The proliferation induced by the phosphatide was of epithelioid cells and epithelioid giant cells as the predominating fundamental units, and the different combinations of the two giving great variety to the reaction. In the case of the lipoidal substances, the phospholipin yielded not only the massive lesions but the greatest variety in different combinations of epithelioid cells and giant cells.

On Plate 3, Fig. 9 (R 158) is shown one of the densest lesions. It is a small part of an extensive, solid mass of epithelioid cells with only a few giant cells; two are shown, one along the upper border and the other near the upper left corner. At the bottom of the figure is a large area of lymphocytes around blood vessels. Between the epithelioid cells are short lines of lymphocytes. The tissue shows practically no free leucocytes. Except at one end the tissue is poor in vessels.

A large mass of tubercular tissue, at a low magnification, is shown in Fig. 10, Rabbit R 153. In this tissue there are definite small clumps of epithelioid cells simulating tubercles; the main mass contains a large percentage of epithelioid giant cells and there are two masses of lymphocytes. Fig. 11, Plate 4, from the omentum of the same animal shows a combination of tubercular granulation tissue and small clumps of epithelioid giant cells. In the upper part is a mass of giant cells infiltrated with lymphocytes in tissue quite richly vascularized, while below is a zone of scattered epithelioid cells. These are quite clearly the typical form with dense cytoplasm. There are no free leucocytes. A lesion of tubercular granulation tissue, with almost no tendency to clumping, is shown in Fig. 12 (R 153). A lesion made wholly of epithelioid giant cells is shown in Fig. 13 from a small lymph node along the internal mammary vein of Rabbit R 319, which had twelve intraperitoneal injections of the phospholipin. At autopsy this lymph node looked like a mass of the specific tissue and was infiltrated with giant cells which in certain areas had almost replaced the follicles and the lymph cords. Such cells were not found in the sinuses. There occurred but little transportation of the phosphatide following these intraperitoneal injections; in one animal (R 158) the specific tissue was found in the mesenteric lymph node; in another (R 319) in a thoracic node; in a third (R 1016) also in the thoracic nodes and the ventral pericardium; otherwise, the reaction has been restricted to the peritoneum.

The study of the chemically produced tubercular tissue offers a chance to study caseation in a simplified form. There have been differences of opinion as to whether leucocytes in caseous material are a response to necrosis or represent a secondary infection. Medlar (15) has recently emphasized the invasion of tuberculous tissue with leucocytes in the absence of secondary infection, an occurrence which is readily explained by the reactions to the chemical fractions from the bacillus. In the response to the various fractions, the phosphatide has been found to possess only a limited power to call out leucocytes into the tissues, leucocytes responding actively only to the first dose. With other substances, however, the undiluted fatty acids and the polysaccharides, for example, each succeeding dose is as irritating as the first. From these observations, it might be inferred that during the disintegration of tubercle bacilli in the tissues, the number of free

leucocytes might vary greatly in accordance with the extremely complex chemical process of disintegration of bacilli and tissues, but that the presence of some free leucocytes might be expected. Auclair (16) reported caseation in rabbits and guinea pigs, produced by lipoids from the tubercle bacillus, but Ray and Shipman (6), in the study of ether-soluble lipoids, considered the areas of necrosis to be only ordinary pus.

In the tubercular tissue produced by the phosphatide, a certain amount of necrosis develops which cannot be discriminated histologically from caseation.

After twelve doses there have always been a few small, yellow nodules to be seen in the gross filled with a cheesy *débris* different in appearance from the thick, white pus of rabbits. These nodules have had a center of necrotic tissue usually surrounded by a border of granulation tissue. The process, however, is much better studied in tiny microscopic nodules where the cells show only early signs of damage instead of disintegration. The sections of tubercular tissue procured after twelve doses of the phosphatide, consisted mostly of masses of epithelioid cells, with hardly a free leucocyte. Here and there were small areas of damaged epithelioid cells surrounded by free leucocytes. The absence of any signs of a general inflammatory reaction in the tissue suggests that the death of epithelioid cells is the initial process, but it is probable that the invasion with leucocytes takes place so quickly as to make the two processes almost synchronous. Nodules can be found so small that the outline of each damaged epithelioid cell can still be seen. There is almost no phagocytosis of leucocytes in such foci, but occasionally a single leucocyte is found engulfed. Fig. 14, depicting a small caseous focus from a lymph node of Rabbit R 319, shows the next stage in the process. The epithelioid cells of the border are markedly vacuolated with only an occasional leucocyte among them; the center is a mass of *débris* in which nuclei of epithelioid cells and leucocytes can be made out. These observations lead to the view that simple caseation is a process in which dead epithelioid cells are invaded and changed by leucocytes.

A process entirely different from caseation has been shown by Doan and Sabin (17) to take place in the regression of tubercles in the bone marrow of rabbits with bovine tuberculosis. The bone marrow always became involved with extensive tubercles after massive (1 to 2 mg.) intravenous injections of the bovine strain, but the lesions did not persist there. The individual epithelioid cells disintegrated and the *débris* was taken up by clasmatoctes. In Fig. 5 of the paper referred to, dying epithelioid cells can be seen in an area of developing red blood cells, without either myelocytes or leucocytes in the neighborhood.

The fate of the tubercular tissue was followed in the last three rabbits of Table II. From the work of Prudden and Hodenpyl (18, 19), it has been known that the tissue produced by dead tubercle bacilli is gradually resorbed.

Rabbits R 368, R 377, and R 379 all received 13 intraperitoneal injections of 80 mg. of phosphatide, a dosage which was proved at autopsy to have produced massive tubercular tissue in the peritoneum of other rabbits of the same lot. Rabbit R 368 was killed 1 month, R 377, 3 months, and R 379, 5 months after the last injection. All three remained in excellent condition throughout the period of observation, gaining in weight. All had shown monocytes as many as 20 percent early in the experiment. During the phase of resorption no anemia developed. After 1 month (R 368), the omentum was still greatly thickened and there were many of the pink nodules of tubercular tissue. The most striking point was the marked vascular organization of the tissue; there were still scattered epithelioid cells. There were adhesions in this animal between the omentum and the intestine and between the body wall and the intestine at the point where the injections had been made, so that there may have been some infection. The amount of tubercular tissue remaining at 3 months was intermediate in amount between that at 1 and 5 months. In R 377, the omentum was still thickened, but much of the bulk was due to the return of fat. Supravitaly there were clumps of epithelioid and giant cells, and an occasional deep pink nodule was found. It consisted almost entirely of epithelioid cells. In R 379 the omentum after 5 months showed an increase in fibrous tissue, but there was no fibrous capsule around the very few remaining tubercular nodules. In this tissue, adventitial cells, loaded with cellular debris, marked out the blood vessels as well as an artificial injection would have done. These three animals thus showed different stages in the disappearance of the chemically produced tubercular tissue, the diffuse reaction disappearing first. After 5 months, only one or two nodules of the specific tissue were still present in the omentum.

From our observations, then, it appears that disintegrating epithelioid cells may be rendered caseous by the infiltration of leucocytes or may be phagocytized by clasmatoocytes without caseation. It is probable that the regression of the chemically produced tubercular tissue is accomplished largely by the latter process, as is that in the bone marrow in the disease itself, since caseation is found in this organ only occasionally.

SUMMARY

The immediate response of the tissues to the injection of the phosphatide A-3 indicates that the material is phagocytized by connective

tissue cells which then appear highly vacuolated. Epithelioid cells appear after three doses, perhaps only after the phospholipin has been to some extent broken down by cells. The increase in tissue occurs largely through the maturation of new monocytes and their transformation into epithelioid cells. During the 2nd week, following repeated daily injections, or after one massive dose, there is an extensive production of epithelioid cells and giant cells, forming typical tubercular tissue in the peritoneum. This tissue shows great variations, consisting either of diffuse granulation tissue, multiple small nodules, or massive aggregations of epithelioid cells. Giant cells are mainly of the simple epithelioid type; the tissues may show only an occasional giant cell or they may predominate or be indeed the only cell present.

Some of the varieties in the types of tubercular tissue produced have been illustrated in the photographs; these figures do not, however, cover the whole range of combinations. The tissue has a greater tendency to vascularization than in the reaction of the actual disease. Some of the small clumps of epithelioid cells are non-vascular; others of the same size possess vessels. The tissue undergoes complete resorption if given sufficient time, in small part by caseation, but to a greater extent probably through the death of the epithelioid cells and the elimination of the débris by the phagocytic action of clasmatoocytes.

The general discussion of the effects of the lipoids and of the sub-fractions from them is given on pages 60 to 69.

REACTION TO THE FATTY ACIDS, THE WAXES, AND THE GLYCERIDES

As shown in Table I (p. 7), the phosphatide was broken down by Dr. Anderson (9) into six simpler substances: oleic acid, palmitic acid, glycerophosphoric acid, glucose, a sugar acid, and a hitherto unknown liquid saturated fatty acid, designated fatty acid I. The first three substances failed to produce tubercular tissue; all of them, however, were irritating, and caused an emigration from the blood vessels of neutrophilic leucocytes, which subsequently suffered phagocytosis by clasmatoocytes, while the administration of the palmitic acid was followed by a considerable reaction of normal monocytes. The glucose and the sugar acid have not been tested.

Effects of the Fatty Acid I

Fatty acid I is a clear, colorless, heavy oil. It is thermostable and was sterilized by heat before being used for the injections. Since Dr. Anderson has given us a part of the original material for biological testing, he has not yet had enough of this acid for complete analysis; he considers, however, that it is a mixture of two or more fatty acids, which possibly differ in their optical activity.

The fatty acid I, in contrast to the other phosphatide subfractions, gave an extensive formation of tubercular tissue (Plate 5), much of it in the massive reaction characteristic of the phosphatide, but more in the form of tubercle-like aggregations of epithelioid cells, with some epithelioid giant cells, the tissue closely resembling that of the actual infection. It thus appears that in the phosphatide the active principle for the production of typical tubercular tissue is carried by this fatty acid. These two substances, the phosphatide and its saturated fatty acid, are far more potent in producing tubercular tissue than the other principal fractions of the lipoids, namely, the so-called waxes and glycerides, and their partitions.

There is no accurate way of determining the total amount of tubercular tissue produced by any fraction, spread out as it is over a wide surface underneath the peritoneal serosa and in the omentum. For the purposes of comparison we may designate the amount produced by the phosphatide + + + +; the amount of tissue resulting from its fatty acid is almost, if not quite, as great, and might be indicated by the same symbol.

Fifteen rabbits have been tested with this acid intraperitoneally, as is shown in Table III.

The dose given, 0.1 cc. (16 mg.), was the amount of the fatty acid contained in 80 mg. of the phosphatide. The fatty acid was first injected undiluted into the peritoneal cavity; seven animals were used in this experiment. Rabbit R 397 received one dose of 16 mg. intraperitoneally and was studied 24 hours later. The abdominal wall showed some hyperemia at the site of the injection, as did the underlying intestine. The omentum was stimulated, with accentuated milk spots, and showed the same pink color as with the phosphatide. In the supravital examination the most striking point was the great number of monocytes; many of these monocytes were young forms and in some areas they were by actual count the predominating cell. In the preparations there could be no confusion between

TABLE III

Protocols

Effects of Intraperitoneal Injections of Fatty Acid I, from Tubercle Bacillus, H-37, in 15 Rabbits

No. of rabbit	Dose	Diluting menstruum	Results
R 397	1 of 16 mg.	None	Accentuated milk spots; dilated vessels; neutrophilic leucocytes; young monocytes; clasmato-cytes not active
R 399	2 of 16 mg.	None	Omentum more stimulated than in R 397; leucocytes being phagocytized by clasmatocytes. Serosal cells irritated. Few epithelioid cells
R 445			Marked hyperemia of body wall, and intestine adherent; may have been small perforation; some monocytes; marked stimulation of clasmatocytes and serosal cells
R 401	3 of 16 mg.	None	Omentum increased milk spots; in some areas abscesses and zones of necrosis. Elsewhere relatively little infiltration of neutrophilic leucocytes but great increase in monocytes and epithelioid cells. Border of mesenteric lymph node of tubercular tissue
R 446			Cecum and colon covered with exudate and adherent. Supravitaly marked maturation of monocytes but signs of irritation predominate
R 619	10 of 16 mg. every 24 hrs.	None	Dense nodular adhesions of cecum and colon to body wall; surface of small intestine and rest of body wall smooth and glistening. Extensive cell division in mononuclear forms and some epithelioid cells. Serosal cells of omentum filled with refractile bodies
R 620	5 of 16 mg. every 48 hrs.		Dense adhesions between cecum and body wall and other loops of intestine with white nodules (pus); serosal cells of omentum filled with refractile bodies; clasmatocytes loaded with cellular debris; marked increase in monocytes; few epithelioid cells and giant cells
R 334	15 of 20 mg.	5 cc. olive oil	All abdominal organs matted together with adhesions. Cultures negative. Supravitaly no normal connective tissue cells. Marked stimulation of clasmatocytes. Remarkable for lymphocytes and for giant cells; some epithelioid giant cells and some foreign body types, 20 to 50 per field. Extreme variation in tissues

TABLE III—*Concluded*

No. of rabbit	Dose	Diluting menstruum	Results
R 335	15 of 20 mg.	5 cc. olive oil	All abdominal organs matted together with adhesions; cultures negative. Remarkable for lymphocytes and for giant cells, predominantly very large foreign body types with many nuclei. Some epithelioid cells. Tissue fibrous and not densely cellular
R 336			Died, peritonitis, colon bacillus. Moderate number of giant cells predominantly foreign body types. Tissues very cellular; neutrophilic leucocytes; clasmatocytes; epithelioid cells
R 337			No peritonitis; cultures negative. Omentum and mesentery thickened and looked like tissue from the phosphatide. Remarkable for lymphocytes and for giant cells, predominantly of foreign body type. Some epithelioid cells
R 510	3 of 16 mg.	0.5 cc. mineral oil	Peritonitis; wall of cecum, omentum, capsule of liver and spleen showed white nodules of pus. In the tissues free neutrophilic leucocytes predominated; a few were in clasmatocytes. There had been a considerable maturation of monocytes, and supravitaly a few epithelioid cells were identified
R 502	12 of 16 mg.	0.5 cc. mineral oil	Adhesions between cecum and body wall. Median lobe of liver and spleen had thickened capsule. Omentum massively thickened, pink, nodular. Epithelioid cells, mainly in tubercle-like structures with lymphocytes. One massive, extensive lesion. Few epithelioid giant cells. Neutrophilic leucocytes in small foci. Extensive replacement of the adipose tissue. No free fluid
R 613			Entire peritoneum involved with pink, nodular tissue. Omentum massively thickened. Epithelioid cells, largely in tubercle-like structures with lymphocytes, but more tubercular granulation tissue than in R 502. Few epithelioid giant cells. Partial replacement of adipose tissue. No free fluid
R 614			Entire peritoneum involved with pink, nodular tissue. Omentum, many small tubercle-like structures with and without lymphocytes. Reaction most massive around reproductive organs. Few epithelioid giant cells; neutrophilic leucocytes in small foci

clasmotocytes and monocytes, since all of the former were large, mature, branched, unstimulated cells, while the monocytes were all smaller, round cells with typical rosettes. The mesentery also showed young monocytes and mature unstimulated clasmotocytes. There were extensive infiltrations with neutrophilic leucocytes, which in fixed sections appeared in the form of multiple small abscesses as the major reaction. The sections showed small clumps of basophilic mononuclear cells, but this reaction would probably not have been recognized without the records of the supravital studies.

Rabbit R 399, which received two injections, had the same accentuation of milk spots as the preceding animal, except that there were a few white nodules of pus large enough to be seen in the gross. Supravitaly interesting changes were seen. There was the same marked irritation shown by the leucocytes; the clasmotocytes, however, instead of being unstimulated, were now active in engulfing the damaged leucocytes, and among the monocytic strain there were some epithelioid cells. The bone marrow showed a shift toward B-myelocytes, indicating the extent of the drain of the leucocytes from the blood stream.

Rabbit R 445 also received two doses. The intestine was adherent to the body wall and may have been perforated. Supravitaly there was observed a marked stimulation both of serosal cells, as indicated by an increase of refractile bodies in them, and of clasmotocytes as shown by increased phagocytosis. There were some monocytes and a few epithelioid cells, but the most striking reaction was the acute peritonitis.

Two rabbits, R 401 and R 446, were given three injections. In R 446, signs of irritation predominated, for the cecum and colon were covered with exudate and had fresh adhesions. In the omentum there had been an active maturation of monocytes, but this reaction was not as striking as the infiltration with leucocytes and the activity of the clasmotocytes. In R 401, on the other hand, there was not much general irritation. In the omentum there were some abscesses and a few zones of necrosis, containing dead mononuclear cells staining heavily in eosin, and many granulocytes, both neutrophilic and eosinophilic; but elsewhere there was but slight infiltration with leucocytes, and the reaction, both as seen supravitaly and in sections, was predominantly monocytic, with many epithelioid cells. Some of these had more than one nucleus showing a tendency toward giant cell formation, but they were not much larger than epithelioid cells. In sections the monocytic and epithelioid reaction was sufficiently definite and striking to be noted, even if the supravital studies had not called attention to it. The tissue did not look like the tubercular granulation tissue, nor like the tubercle-like masses illustrated for the phosphatide, for there had been no great thickening of the omentum, no great increase in blood vessels; but in a relatively normal omentum there was this marked increase in the one strain of cells. Very few plasma cells were found, but there were many clumps of deeply basophilic mononuclear cells, as well as typical monocytes and epithelioids, some of them in division, both scattered and in small clumps of 8 to 10 cells.

The acute lesions produced in experiments with from one to three doses demonstrated the extremely irritating character of the undiluted fatty acid. Except in one rabbit (R 445) in which the wall of the intestine may have been punctured by the injection needle, it was judged that the irritation was due to the acid itself and not to injury or infection. There was considerable evidence of a stimulus toward monocytes, especially plain in Rabbit R 401. There were none of the large vacuolated cells that characterized the early reaction to the phosphatide, which were regarded as probably indicating the phagocytosis of the phosphatide, but rather a tendency was observed to a direct maturation of monocytes from more primitive forms.

The remaining animals receiving fatty acid I were given from 3 to 15 repeated doses, and, with one exception (R 502), studies of the peripheral blood were made. The changes in the blood cells (Table IV) were not marked. All the animals showed a slight drop in the number of red blood cells and in hemoglobin, but the bone marrow revealed no obvious changes. In two animals, R 613 and R 620, there was a moderate leucocytosis due to a rise in neutrophilic leucocytes; the monocytes tended to increase and the lymphocytes to decrease in the majority of the rabbits, the most striking changes being noted in R 620, R 334, and R 337.

Two of the rabbits (R 619 and R 620) were given 10 and 5 intraperitoneal injections of 16 mg. of undiluted fatty acid I. In both the irritation was extreme, the intestine, especially the cecum, being bound to the body wall. The intestine was covered with fibrin and exudate; the omentum and mesentery were extensively involved with a reaction of extreme complexity. There was an increase in fibrous tissue, most marked in R 619, making many adhesions; there were many areas of necrosis in some of which were great masses of damaged cells, looking like epithelioids (R 620); in the border of these areas, clasmatoocytes, loaded with cellular debris, were present in such numbers as to make them the predominating cell of the reaction. There were masses of granulation tissue, markedly cellular and richly vascularized. Scattered diffusely in this tissue were epithelioid cells and epithelioid giant cells, either singly or in small clumps, but not in sufficient numbers to make typical tubercular granulation tissue. This was interesting in view of the fact that this same fatty acid, when subsequently diluted with a bland oil, gave a reaction so closely like tuberculosis that it could be discriminated only by the entire absence of tubercle bacilli (Plate 5). With the undiluted acid, however, in repeated doses, the extreme irritative reaction appeared to minimize and certainly obscured the reaction toward tubercular tissue. In the omentum the fat cells were in process of changing, which also added to the confusion of the picture. In Rabbit R 620 there were small nodules of epithelioid cells in a Peyer's patch; in neither animal were the mesenteric nodes involved.

Olive oil, 5 cc. per dose, was first tried as a menstruum to dilute the fatty acid

TABLE IV

Blood Counts in Eight Rabbits Receiving 10-14 Intraperitoneal Injections of Fatty Acid I

No. of rabbit	Original white blood cell count and neutrophils (per c.mm.)	Highest white blood cell count and neutrophils (per c.mm.)	Original red blood cells (per c.mm.) and hemoglobin	Final red blood cells (per c.mm.) and hemoglobin	Monocytes		Lymphocytes	
					Originally	Highest	Originally	Highest*
R 334	12,300 3,936	13,300 9,310	5,930,000 61%	4,950,000 49%	1,476	1,862	6,150	1,596
R 335	10,300 4,841	10,900 8,502	6,280,000 59%	5,240,000 53%	206	1,599	4,017	6,150
R 336	10,700 4,387	14,200 6,248	5,460,000 60%	4,880,000 50%	1,177	1,344	4,173	2,184
R 337	11,800 4,956	11,800 9,086	6,340,000 72%	5,740,000 60%	1,584	1,416	3,776	944
R 613	6,850 2,329	22,300 17,171	5,190,000 58%	4,690,000 55%	685	1,666	2,877	1,368
R 614	7,600 2,736	8,700 2,523	5,570,000 69%	4,940,000 69%	684	1,914	3,572	2,784
R 619	6,500 2,950	10,450 5,956	5,180,000 73%	4,360,000 67%	455	1,202	2,340	2,682
R 620	5,850 2,808	20,100 10,653	4,990,000 73%	4,300,000 Lost	234	5,226	2,691	2,211

* At time of highest monocytes.

(20 mg.) in four animals, R 334, R 335, R 336, R 337, while at the same time five animals as controls were given the same number of injections of the olive oil alone. The irritation produced with the combination was much more marked than with the undiluted fatty acid, and the controls demonstrated the very marked tendency of olive oil to produce foreign body giant cells. In three rabbits, R 334, R 335, and R 336, all the abdominal viscera were matted together with dense adhesions; one, R 336, died and the colon bacillus was recovered as a probable participant in the general peritonitis. All the tissues were remarkable for the great numbers of giant cells, of both types, but with the large foreign body forms predominating; fifty per low power field were not uncommon numbers, and many of them were vacuolated. Next to the giant cells, multiple small masses of lymphocytes were the most striking part of the reaction, especially in R 334, R 335, and R 337. Lymphocytes were not a feature of the controls with olive oil alone. The tissues were markedly uneven; there was much granulation tissue, and in some areas new connective tissue cells were closely packed together. In other places the tissue was open. In R 334, supravitaly studied, not a single normal connective tissue cell was seen. The clasmatoocytes were stimulated; there were some nests of epithelioid cells, most marked in R 337, and all the tissues showed epithelioid giant cells. Nevertheless, the sections did not simulate the lesions of tuberculosis. Yet the group, as a whole, tended to show more epithelioid cells and Langhans' giant cells than the controls with olive oil alone.

The results with repeated doses of the undiluted fatty acid and with repeated doses of the fatty acid mixed with olive oil were not as suggestive of tuberculous tissue as those with the few doses of the undiluted acid, in which a maturation of monocytes had been so marked. It was clear that the undiluted acid was too irritating for repeated doses, and that with olive oil the extreme reaction of the menstruum itself toward foreign body giant cells obscured the tendency toward monocytes. Therefore a series of experiments were undertaken to find if possible a relatively inert oil, with the result that a pure mineral oil (Nujol) was eventually selected for use. It proved to be an excellent menstruum. It was practically inert in one, two, and three doses; and with twelve injections, its effects were so much simpler and so much less extensive than with other menstrua tried, and especially the reaction toward epithelioid cells was so very slight that the oil has been used during the past year as a general diluting medium for the fatty acids studied. It has also been used as a solvent for the waxes and their derivatives.

In Fig. 19 on Plate 6 there is, however, shown a film of omentum, supravitaly stained with neutral red and photographed while the cells were living, to illustrate the relative uniformity of the reaction to twelve intraperitoneal injections of mineral oil (Rabbit R 661). There had been an increase in number of fibroblasts and clasmatocytes. This film is to be compared with a similar film of omentum photographed while the cells were alive, after eleven doses of the phosphatide (Plate 1, Fig. 4). In sections there were a few giant cells, predominately of the foreign body type, and an occasional epithelioid cell.

One rabbit, R 510, given three injections of the fatty acid in mineral oil, showed a general peritonitis. In spite of the great infiltration of the tissues with leucocytes and zones of necrosis, the omentum showed areas filled with monocytes; in the fresh tissues a few typical epithelioid cells and giant cells derived from them were seen, and they can be identified in sections.

Three rabbits (R 502, R 613, and R 614) were given twelve intraperitoneal injections each of 16 mg. of fatty acid I in 0.5 cc. mineral oil. Rabbit R 513, with mineral oil alone, was the specific control for R 502, and Rabbits R 621 and R 622 for the others. The reaction in these rabbits and their controls, as seen in the gross specimens at autopsy, made the most striking contrast; in each case the reaction in the control rabbit with mineral oil alone was slight, as compared with the massive production of new tissue in the rabbits with the fatty acid.

Instead of an excess of free oil in the peritoneal cavity, which is always present after administration of the mineral oil alone in these doses, the rabbits with the fatty acid plus mineral oil had a peritoneum so dry that no peritoneal fluid could be obtained for the study of the free cells. All three rabbits showed an extensive development of tubercular tissue. There was an extensive involvement of the peritoneum; the omentum, the entire wall of the cecum, the diaphragm, the capsule of liver and spleen, and the connective tissue around the reproductive organs were massively involved with new tissue, pink in color like that from the phosphatide. In some zones this new tissue was in thick, dense layers; in others it was in nodules. There was never as complete an absence of neutrophilic leucocytes in the tissues as after repeated injections of the phosphatide; some leucocytes, both scattered and in small foci, were present in all the sections, but they formed a minor part of the reaction in comparison to the epithelioid cells.

The tissue reaction of Rabbit R 502 is represented by a quite characteristic tubercle-like focus taken from the omentum (Plate 5, Fig. 15). It is densely surrounded by lymphocytes; there were no giant cells in this area but a few of both types were found in the sections. In the wall of the cecum of this animal were extensive layers of epithelioid cells as large and as dense as in Plate 3, Fig. 9, from the phosphatide. In some of the blocks fixed from the omentum and from the capsule of the spleen there were large abscesses which had no relation to caseation. They were simply masses of leucocytes surrounded by granulation tissue. This granulation tissue, richly vascularized, had only a minor infiltration with epithelioid cells, and there were but a few small clumps of epithelioid cells in the neighborhood.

Rabbit R 613 showed the same extensive formation of specific tubercular tissue, widely distributed in the peritoneum, but especially massive in the mesentery and the omentum. Figs. 16 and 17 on Plate 5 show the reaction in the omentum. In Fig. 16 are three tubercle-like bodies with two large giant cells, probably of the rosette type, but possibly atypical, through the massing of the nuclei at one end. There is a dense capsule of lymphocytes. Fig. 17 shows tubercular granulation tissue, more dense in the lower part of the section. Along the upper border is a tiny, tubercle-like clump of epithelioid cells, and on the left border is a typical rosette giant cell. The tissues of Rabbit R 614 are represented in Fig. 18, which again shows small and larger collections of epithelioid cells with some diffuse infiltration of lymphocytes. In this animal the tissue was especially massive on the posterior body wall and around the reproductive organs. There were more epithelioid giant cells in this animal, but never as many as with the phosphatide.

It will be seen that all three of the rabbits receiving twelve doses of the fatty acid I, diluted with a bland oil, showed an extensive production of tubercular tissue, some of it as massive as with the phosphatide, but much more in the form of clumps of epithelioid cells, making circumscribed, tubercle-like formations, similar to those of the infection. There was only a moderate tendency toward epithelioid giant cells. There were some tubercular granulations, but a comparison of Plate 5 with the plates of tissues from the phosphatide will indicate that there was less variation in the type of specific reaction with this fatty acid than with the phosphatide, that is, the lesions were more uniform. There was, of course, a considerable increase in non-specific connective tissue, to make a framework for the tubercle-like structures, but this interstitial tissue lacked the extreme reaction found with the undiluted acid. In general, this fatty acid gave a reaction, when its irritative effects were minimized by dilution in mineral oil, which simulated the actual disease more closely than any other part of the lipoids; it was almost the same as the phosphatide from which it was derived, in epithelioid formation, but had more of the reaction in the form of discrete tubercles.

Effects of the Purified Wax and Its Derivatives

In Table I it will be seen that there were two groups of fractions which were soluble in chloroform and insoluble in acetone. Dr. Anderson has designated these substances "purified wax" and "soft wax," though he does not regard them as true waxes in the chemical sense (9).

The purified wax is a white, very light powder. From it Dr. Anderson obtained an unsaponifiable substance and a second liquid, saturated fatty acid. All three of these fractions had to be given in mineral oil. The purified wax could not be rubbed into a suspension in distilled water, as was done with the phosphatide; even less could the unsaponifiable substance, which floated as a dry, feathery substance on top of the water. Both of them were readily soluble in mineral oil. The purified wax and its fatty acid II gave reactions which we estimate as being positive for the specific formation of tubercular tissue; nevertheless, the reaction was smaller in amount than that of the phosphatide and fatty acid I. We estimate the amount of this reaction as ++ in comparison with the ++++ of the phosphatide. It was therefore necessary to make a careful comparison of the reaction of the purified wax and fatty acid II with that from the injection of the mineral oil alone. Beside this moderate reaction toward monocytes, epithelioid cells, and Langhans' giant cells (Plate 6, Figs. 20-23), the purified wax and fatty acid II gave a marked increase in general connective tissue, which is also found in tuberculosis. The unsaponifiable substance gave only an increase in general connective tissue cells.

The Purified Wax.—Five rabbits were tested with the purified wax intraperitoneally, as is shown in Table V.

Rabbit R 455 had one dose of 20 mg. of the purified wax, with 0.75 cc. of oleic acid as a diluting menstruum, and the tissues were studied 2 days later. The result was a marked irritation, and the tissues showed many abscesses. There was some phagocytic activity of the clasmatoocytes and in the supravital studies some monocytes were seen, but they were few compared to the leucocytes. Oleic acid alone was found to be very irritating to the tissues and so was abandoned as a diluting menstruum.

Given in mineral oil, the material was much less irritating; in rabbits with few doses, R 550 with two, and R 551 with three, there were some neutrophilic leucocytes and some activity of clasmatoocytes in destroying them, but there was a striking increase in monocytes, with a few epithelioid cells in R 551, and this strain of cells showed active division in sections. This tissue on the whole did not simulate that of tuberculosis, and yet the increase in monocytes and the occasional epithelioid cell would be described as the most striking reaction.

Two rabbits, R 937 and R 938, received twelve intraperitoneal doses of 16 mg. of the purified wax in 0.5 cc. of mineral oil. At autopsy, 24 hours after the last dose, a marked thickening of the omentum was found; the diaphragm, the capsules of liver and spleen, and the wall of the cecum were also involved. In Rabbit R

TABLE V

*Protocols**Effects of Intraperitoneal Injections of the Purified Wax and Its Derivatives, the Unsaponifiable Substance and Fatty Acid II*

No. of rabbit	Dose	Diluting menstruum	Results
Purified wax			
R 455	1 of 20 mg.	0.75 cc. oleic acid	Marked irritation, peritonitis; many white nodules of pus. Clasmatocytes active in phagocytosis; many monocytes
R 550	2 of 16 mg.	0.5 cc. mineral oil	Omentum stimulated; some free neutrophilic leucocytes, many engulfed in clasmatocytes; many monocytes, scattered and in small clumps in active division. A few epithelioids
R 551	3 of 16 mg.		Omentum stimulated; more free leucocytes than in R 550. Clasmatocytes active. Many monocytes in division. Occasional epithelioid giant cell
R 937	12 of 16 mg.		No peritonitis; serosa smooth and glistening. Omentum, surface of diaphragm, and capsule of spleen massively thickened. Tissue edematous, red with white spots; looked like tuberculosis. Marked increase in general cellular connective tissue, with a few tubercle-like bodies and typical Langhans' giant cells. Some foreign body giant cells. A few plasma cells
R 938			No peritonitis; serosa smooth and glistening. Distribution of new tissue as in R 937. Omentum massive with general connective tissue. A few nodules of monocytes. Many cells difficult to classify because they have engulfed so much oil. Tissues look more like reaction to unsaponifiable substance
The unsaponifiable substance			
R 548	2 of 16 mg.	0.5 cc. mineral oil	Serosa smooth and glistening. Omentum not thickened but showed both neutrophilic and eosinophilic leucocytes; some phagocytosis of them by clasmatocytes. A few monocytes
R 549	3 of 16 mg.		No peritonitis. Omentum normal, except that serosal cells were stimulated. Some milk spots increased in density. No leucocytes. Few monocytes

TABLE V—*Concluded*

No. of rabbit	Dose	Diluting menstruum	Results
The unsaponifiable substance— <i>Concluded</i>			
R 945	12 of 16 mg.	0.5 cc. mineral oil	No peritonitis. Marked increase in connective tissue in omentum, capsule of liver and spleen, and over the diaphragm. Few free leucocytes in R 945; many clumps of lymphocytes; many monocytes; no epithelioid cells; moderate number of foreign body giant cells. Very few Langhans' giant cells. Few plasma cells
R 946			
Fatty acid II			
R 539	2 of 16 mg.	0.5 cc. mineral oil	Omentum showed accentuation of milk spots due to emigration of leucocytes and maturation of monocytes
R 540	3 of 16 mg.		Omentum: large dense milk spots due to emigration of leucocytes and to monocytes, epithelioid cells, and few epithelioid giant cells. Occasional foreign body giant cell. Marked dilatation of vessels. Sections show some clumps of monocytes
R 615	12 of 16 mg. every 24 hrs.		Parietal peritoneum, omentum, mesentery, and capsule of spleen and liver massively thickened. Omentum so cellular and dense, difficult to make films. Many clumps of monocytes with occasional epithelioid cell. Typical Langhans' giant cells
R 616	12 of 16 mg. every 48 hrs.		Omentum shows large milk spots in central area; rest massively thickened. Leucocytes and stimulated clasmatocytes. Clumps of cells were predominately epithelioid cells, rather than monocytes as in R 615

937, the tissue had, in the gross, the appearance of tuberculosis; it was swollen and edematous, was pink in color, and showed many white spots. In supravital preparations it appeared that there had been a marked new formation of undifferentiated connective tissue cells; these were of medium size, round, and showed less reaction to neutral red than do fibroblasts, clasmotocytes, or monocytes. Beside these undifferentiated cells, there had been a considerable development of epithelioid cells with the typical rosettes of fine bodies and many Langhans' giant cells, which predominated in number over the foreign body giant cells which were also present. There was an increase in young monocytes and the amount of the

specific reaction, that is, of epithelioid cells and epithelioid giant cells, was sufficient to place the purified wax in the group of the active lipoidal substances.

The tissues of Rabbit R 938 were likewise much thickened; supravitaly the reaction was mixed and the specific reaction less in amount. Sections of the material showed a great increase in new indifferent connective tissue cells; these appeared as masses of nuclei without distinct cellular outlines. The masses of nuclei were taken to represent the clumps of undifferentiated cells in the fresh preparation, where the cellular outlines were sharp. Such masses of nuclei under low power suggest tubercles only vaguely and at higher powers not at all. In general the sections would not have been called characteristic of tuberculosis, because the epithelioid cells, both those that occurred alone and those in small clumps, were so scattered, and the indifferent connective tissue with a great increase in nuclei predominated to such an extent. Giant cells numbered from ten to twenty per low power field of the microscope. Some of them were typical of the rosette type with peripheral nuclei, but more of them looked like the non-rosette forms; most of them had taken in large or small droplets of the mineral oil which made them atypical. The epithelioid cells were in part typical, as was seen by the supravital technique, when their rosettes had the fine bodies, but many of them in sections appeared vacuolated, probably also from the presence of the mineral oil in medium sized droplets. There were many scattered monocytes. In places the small clumps of epithelioid cells were surrounded by lymphocytes; in others lymphocytes were lacking. On the diaphragm there were several quite definite tubercle-like structures. In general, though there was distinctly more of a reaction toward the formation of epithelioid cells and Langhans' giant cells than in the controls with mineral oil alone, this portion of the reaction was minimal as contrasted with the production of indifferent cells in the connective tissue itself. The sections from Rabbit R 938 were similar to those from R 937, except that they showed fewer epithelioid cells and giant cells.

The reaction to the purified wax was thus considered to be ++ in contrast to the ++++ of the phosphatide, as far as the production of epithelioid cells and Langhans' giant cells was concerned. The reverse would be the case if the amount of increase in general cellular connective tissue were appraised. The predominant reaction was of indifferent connective tissue cells, and there was in both rabbits, R 937 and R 938, a diffuse reaction of plasma cells in moderate numbers.

The Unsaponifiable Substance.—The unsaponifiable partition of the purified wax—the acid-fast portion of the lipoids—also showed the tendency to cause a marked increase in general connective tissue cells, without any of the stimulus toward the formation of tubercle-like masses of epithelioid cells.

The unsaponifiable substance was given to four rabbits (Table V). Rabbits R 548 and R 549, which received two and three doses of 16 mg. in 0.5 cc. mineral oil, showed only a slight reaction; in both the serosal cells appeared stimulated in the fresh preparations, that is, they showed an increase in small refractile bodies. There were free leucocytes in R 548, both neutrophilic and eosinophilic, and the clasmatocytes showed a slight increase in neutral red bodies; a few clasmatocytes contained leucocytes. Some of the milk spots were almost wholly of monocytes, and sections showed them in clumps, together with a few smaller, more basophilic mononuclear cells. There was an occasional mononuclear cell in division. This reaction was negative for tubercular tissue.

Two rabbits, R 945 and R 946, were then given twelve doses of 16 mg. of the unsaponifiable substance in 0.5 cc. mineral oil, and were studied 24 hours after the last dose. In both animals there had been a marked increase in new tissue, involving the peritoneum in the same areas as with the phosphatide, that is, the omentum, the capsule of liver and spleen, and the diaphragm. The new tissue equalled in amount that produced by the phosphatide; the omentum was just as massive. The cellular reaction, as seen supravitaly and in sections was, however, quite different. There was a marked increase in new connective tissue cells. The milk spots were predominantly of medium sized cells, with a few scattered neutral red bodies. There were many monocytes. Sections showed an increase in general connective tissue cells, with replacement of the fat, and scattered everywhere among these less differentiated cells were diffuse masses of young, very basophilic, mononuclear cells, together with some monocytes and a few plasma cells. There was a moderate amount of cell division in the mononuclear forms. There were a few scattered giant cells, mainly of the foreign body type.

Thus the unsaponifiable material caused a marked increase in non-specific connective tissue cells with some increase in monocytes, but did not produce the specific tubercular reaction of epithelioid cells and epithelioid giant cells.

Fatty Acid II from the Purified Wax.—The fatty acid II caused the production of tubercular tissue in about the same amount as the purified wax, indicating that the entire active principle of the purified wax was carried in this component. The reaction has therefore been designated ++.

The acute reaction was tested in two rabbits, R 539 with two doses of 16 mg. in 0.5 cc. mineral oil, and R 540 with three (Table V). The reaction was of leucocytes and monocytes. The peritoneal exudate reflected this; for example, in R 539, the percentage of leucocytes was 26, of monocytes 68, with the remaining 6 per cent of lymphocytes and serosal cells. The rabbit with three doses, R 540, had a few epithelioid cells among the monocytes.

Two rabbits, R 615 and R 616, were given twelve doses of 16 mg. of fatty acid II in 0.5 cc. mineral oil, the first every day and the second every other day. In both there was a marked increase in new tissue in omentum, mesentery, and under the parietal peritoneum. The omentum was fully as massive as with the phosphatide; there was almost no exudate. The tissue from these rabbits differed from that with the phosphatide and fatty acid I in that only a part of the new tissue was of monocytes and epithelioid cells, and there was a great reaction of non-specific connective tissue cells, undifferentiated in type, and fibroblasts. Thus, as can be seen on Plate 6, Fig. 20, the tissues do not suggest tuberculosis as conclusively as does the reaction to fatty acid I (Plate 5) or to the phosphatide (Plates 1-4). Fig. 20 was taken with a high dry lens at a magnification of 200. It can be seen that the clumps of monocytes and epithelioid cells are so tiny that they would not be conspicuous under a low power lens. The reaction is, however, of typical tubercular granulation tissue. There are scattered lymphocytes, but none in capsules around the tiny clumps of monocytes. The vessels at the right are crowded with lymphocytes. Scattered everywhere throughout this tissue were clumps of cells which on supravital examination were called monocytes, with an occasional epithelioid cell, and a few epithelioid giant cells. This is exactly what can be seen in Fig. 21. Except for the two larger cells, probably epithelioids, in the lower left border and a small Langhans giant cell in the center, this section shows a clump of monocytes; above this is a small epithelioid giant cell and below the clump an especially typical form. In Fig. 22 is another typical epithelioid giant cell, with a central area of necrosis, and the débris of a leucocyte; such an area of necrosis is frequent in the corresponding giant cells of the actual infection. The infiltration of these tissues with monocytes is shown in Figs. 22 and 23. In the latter, one of the cells, probably a monocyte, is in division. The amount of non-specific connective tissue is best seen in Fig. 20. The tissues of Rabbit R 616 were similar, except that there were fewer young monocytes and the tiny clumps of cells, microscopic tubercles, were epithelioid types; in this connection it will be noted that the reaction was of longer duration in this animal, since the injections were given every other day.

Thus, all of the three substances in this group, the purified wax and its derivatives, the unsaponifiable substance and the liquid fatty acid II, contain a stimulus for the production of relatively undifferentiated, connective tissue cells. This is the dominant reaction. Beside this increase in connective tissue cells, the purified wax and the fatty acid II produce some epithelioid cells, showing a rather marked tendency to cause a formation of tiny clumps or tubercles, which, however, are not surrounded by lymphocytes to any extent. Epithelioid giant cells are numerous and typical of those found in the actual infection. The relative amount of this specific tubercular reaction

from the purified wax and fatty acid II may be estimated as ++; and from the unsaponifiable substance as zero.

Effects of the Soft Wax and Its Derivative Fatty Acid III

The "soft wax," which is soluble in chloroform and insoluble in acetone, is a brown, wax-like substance, a soft solid at room temperature, miscible with mineral oil. It yielded on chemical analysis (Anderson, 9) a liquid, saturated fatty acid which has a deeper yellow color than fatty acid I.

The soft wax was given to four rabbits (Table VI). In the acute reaction (Rabbit R 546, two doses of 16 mg. in 0.5 cc. mineral oil, and R 547, three doses) the soft wax proved to be much more irritating than the purified wax. After two doses the serosal cells were stimulated, as shown by refractile bodies in them; the predominating reaction was of leucocytes with clasmatocytes engulfing them. After three doses the serosal cells were filled with refractile bodies and made so dense a membrane that it was hard to focus through them in fresh preparations. In both animals there was a marked dilatation of the blood vessels. After two injections the tissues were massive with leucocytes, but there were some monocytes; after three doses there were fewer leucocytes and more monocytes.

Two rabbits, R 947 and R 948, were given repeated injections. Rabbit R 947 was found dead of pneumonia after the tenth dose; the other one was given twelve doses of 16 mg. each in 0.5 cc. mineral oil. In Rabbit R 947 there was a fresh peritonitis, especially evident over the liver and spleen. Rabbit R 948 showed no infection. In both animals there was a great dilatation of the blood vessels in the new tissue in the omentum and over the abdominal organs. Fig. 24 on Plate 7 is taken from extensive lesions on the abdominal surface of the diaphragm. The lesion measured 2 mm. in thickness in the sections which had undergone the shrinkage of the dehydration. The dilatation and congestion of the veins are a striking factor, as is shown in the photograph. There are two small clumps of epithelioid cells; the larger is at the upper left corner and is infiltrated with lymphocytes, and the smaller is at the lower left corner. The section also showed scattered epithelioid cells. Giant cells were present in the tissues in moderate numbers, some of them typical rosette types; others of the non-rosette type with large vacuoles.

There was a considerable new growth of connective tissue, not as marked, however, as with the purified wax and its derivatives. For example, the fat of the omentum was only partially replaced by connective tissue cells. There was some typical granulation tissue, with many fibroblasts. There were many leucocytes and many of them

TABLE VI

*Protocols**Effects of Intraperitoneal Injections of the Soft Wax and Its Derivative, Fatty Acid III*

No. of rabbit	Dose	Diluting menstruum	Results
Soft wax			
R 546	2 of 16 mg.	0.5 cc. mineral oil	Milk spots of omentum markedly stimulated. Predominating reaction of leucocytes scattered and in small foci. Many clasmatocytes full of yellow debris. Serosal cells full of refractile bodies. Some monocytes. Vessels dilated
R 547	3 of 16 mg.		Some seropurulent fluid. Omentum: extensive stimulation of milk spots and fat replaced; leucocytes; serosal cells markedly irritated. Some monocytes and epithelioid cells in small clumps. Marked dilatation of vessels
R 947	10 of 16 mg.		Died of pneumonia night after 10th dose. Body wall had hemorrhage; no puncture of intestine; marked peritonitis with general fibrinous exudate. Omentum moderately thickened, leucocytes and small tubercle-like clumps. Marked dilatation of vessels
R 948	12 of 16 mg.		Omentum much thickened; diaphragm, liver, and spleen covered with new tissue; extreme dilatation and congestion of vessels (Fig. 24). Many scattered epithelioid cells and tubercle-like structures. Lymphocytes
Fatty acid III			
R 542	2 of 16 mg.	0.5 cc. mineral oil	Omentum: slight increase in milk spots but no thickening. No peritoneal irritation. Few leucocytes, neutrophilic and eosinophilic; predominating reaction clasmatocytes engulfing leucocytes. Few monocytes. No epithelioid cells seen
R 543	3 of 16 mg.		Omentum delicate; no marked thickening; very few leucocytes free and only an occasional one in clasmatocytes. Marked monocytic reaction, many in division. Few epithelioids

TABLE VI—*Concluded*

No. of rabbit	Dose	Diluting menstruum	Results
<i>Fatty acid III—Concluded</i>			
R 617			Adhesions between cecum and body wall. Some thick, ropy, peritoneal exudate. Omentum massively thickened. Leucocytes not marked; few in clasmatoocytes. Predominating reaction of monocytes, epithelioid cells, and Langhans' giant cells. Few plasma cells
R 618	12 of 16 mg.	0.5 cc. mineral oil	Hemorrhage into body wall. Cecum adherent to body wall. Omentum extremely thickened; marked dilatation of vessels. Mesentery also involved. Clasmatoocytes active but predominating reaction of clumps of monocytes and tubercle-like masses of epithelioid cells. Few giant cells. Lymphocytes. Many foci of basophilic mononuclear cells. Few plasma cells

were in clasmatoocytes. In Rabbit R 947 there was a focus of myelocytes in the omentum. Scattered through this tissue were considerable numbers of epithelioid cells; especially over the intestine and the diaphragm there were many more epithelioid cells than in the reaction to the purified wax. Giant cells were present in small numbers, some epithelioid in type, more of the large, multinuclear foreign body types. The reaction includes positive specific tissue which is intermediate in amount between that found following the purified wax and the phosphatide.

Fatty Acid III from the Soft Wax.—Four rabbits were given the fatty acid III, two for the acute reaction (R 542 and R 543) and two (R 617 and R 618) for the effect of twelve repeated doses (Table VI).

After two doses (R 542) the milk spots of the omentum were increased in number but were not much thickened. The tissues showed neutrophilic and eosinophilic leucocytes and some phagocytosis of these by clasmatoocytes, and there was an increase in monocytes. Sections showed a marked activity of the clasmatoocytes in destroying leucocytes, and there was much cell division both of the serosal cells and of free mononuclear cells, the size of monocytes. The monocytes and phagocytic clasmatoocytes were plain both in the folds of the omentum and in the exudate caught between these folds. In Rabbit R 543 the omentum was more thickened;

clasmatocytes were still present, containing leucocytes, but the monocytic reaction had become predominant. The supravital reaction revealed as pure a monocytic response as with any of the lipid fractions; this reaction was clear in the sections, making excellent tissue for the study of this type of mononuclear cell.

Rabbit R 617 received twelve intraperitoneal doses. There was a marked development of new connective tissue; the non-specific connective tissue was less cellular than that produced by the purified wax and its derivatives, but scattered everywhere in it were tubercle-like structures, similar to those from fatty acid II, but more in number and somewhat larger. Some of these clumps of epithelioid cells were densely surrounded by lymphocytes. There were few giant cells, small rosette forms and large foreign body types.

Rabbit R 618 showed the same reaction, but the clumps of epithelioid cells were still larger and more numerous. These lesions are shown in two photographs (Plate 7, Figs. 25 and 26). Multiple tubercle-like structures stained rather faintly are shown in Fig. 25, with a mass of lymphocytes at the upper right border. Fig. 26 is from the mesentery near the mesenteric lymph node; it shows a clump of epithelioid cells and one large giant cell, with a few epithelioid cells near it. The giant cell is complex. It has a peripheral row of nuclei and at one end a clump of nuclei; it had phagocytized a leucocyte and in one zone the cytoplasm is vacuolated. It is probably an atypical epithelioid or Langhans type.

In general the specific reaction caused by fatty acid III was overwhelmingly toward epithelioid cells as compared with giant cells. Only a few giant cells were found; in some sections none. The specific activity of the soft wax and of the fatty acid III derived from it was practically equal in amount, and can be considered as +++ in comparison with that of the phosphatide (++++) and fatty acid II (++).

Glycerides

The acetone-soluble glycerides (Table I) also soluble in alcohol-ether, have not been tested biologically, but we have studied the effects of the saturated fatty acids derived from the glycerides. The mother substance was a brown solid of wax-like consistency. It yielded on analysis two saturated fatty acids, designated IV (Anderson's No. 1) and V (Anderson's No. 2), in Table I. The proportion of fatty acids in the original glyceride, namely, about 40 per cent, was much higher than that of any of the other three primary lipoids. Because of the larger quantities available, Dr. Anderson has been able to separate these two fatty acids by fractional distillation, and has found that each of them is composed of the same two simpler fatty acids, one optically

active and the other optically inactive, only combined in different proportions. He considers that the fatty acids previously described, I, II, and III, are also mixtures of fatty acids which may be separated by fractional distillation when sufficient quantities are available. Fatty acid IV contained more of the optically inactive form, which, on analysis, proved to have the formula $C_{18}H_{32}O_2$. This isomer of stearic acid, Dr. Anderson has named tuberculo-stearic acid. It is an oil at room temperature.

Fatty acid V contained a greater proportion of the optically active acid of the formula $C_{26}H_{52}O_2$. Dr. Anderson has named this fraction phthioic acid. It has a low melting point, 28°C ., and is optically active; $[\alpha]^{20}_D = 7.98^{\circ}$. The tuberculo-stearic and phthioic acids represent the highest chemical purity of any of the fractions thus far tested.

Fatty acids IV and V from the glyceride, and the phthioic acid all produced epithelioid cells and epithelioid giant cells in moderate numbers, while the tuberculo-stearic acid was negative in this reaction, indicating that the active element in all three positive substances is the optically active phthioic acid. With these fatty acids, the epithelioid cells were scattered, as in Fig. 27, with no tendency whatever toward the formation of tubercle-like bodies. In this regard these acids have differed from the other liquid saturated fatty acids, from phosphatide, purified wax, and soft wax, with all of which tubercle-like bodies were formed. The epithelioid cells produced were, however, entirely typical if relatively few in number, as indicated by the symbol + in Table I.

The experiments with these two acids before and after the final purification are summarized in Table VII.

Two rabbits were given twelve intraperitoneal doses of each original mixture in 0.5 cc. mineral oil, but the dosage of the purified forms was in each case only 5 mg. in contrast to 20 mg. of the corresponding fatty acids before purification.

Rabbits R 918 and R 919 received twelve doses of 20 mg. of fatty acid IV. Rabbit R 918 had a great excess of adipose tissue in omentum and mesentery, and the omentum was only moderately thickened with new tissue. On the other hand, the omentum of Rabbit R 919 was markedly thickened. In both animals the most striking reaction was of giant cells, both types being present, but with more of the large, non-rosette types. Both showed some scattered epithelioid cells, none in the form of tubercles, not even in small clumps like those from the purified wax.

TABLE VII

*Protocols**Fatty Acids from the Glycerides*

No. of rabbit	Substance tested	Dose	Diluting menstruum	Results
Fatty acid IV and its major fraction, tuberculo-stearic acid				
R 918	Fatty acid IV	12 of 20 mg.	0.5 cc. mineral oil	Great excess of adipose tissue in peritoneal cavity; omentum only moderately involved with new tissue. Few scattered epithelioid cells showing division; many giant cells, both types, predominantly foreign body; some free leucocytes; plasma cells. Marked dilatation of vessels
R 919				Omentum markedly thickened; few scattered epithelioid cells; many giant cells, predominantly foreign body types; some free leucocytes; plasma cells; marked dilatation of vessels; clumps of myelocytes in omentum
R 980	Tuberculo-stearic acid	12 of 5 mg.		Omentum moderately thickened; milk spots of undifferentiated cells showing less reaction to neutral red than monocytes; occasional epithelioid cells; many foreign body giant cells; some free leucocytes; plasma cells
R 981				Omentum moderately thickened, weight 7 gm.; milk spots showed monocytes and occasional epithelioid cells; many foreign body giant cells; few free leucocytes; plasma cells
Fatty acid V				
R 920	Fatty acid V	9 of 20 mg.	0.5 cc. mineral oil	Died; immediate autopsy. Cause of death not determined. Cirrhosis of liver, large spleen (3 gm.); great excess of adipose tissue in peritoneal cavity; omentum showed some stimulated areas filled with cells, nature of which not determined, size of epithelioids but loaded with refractile bodies

TABLE VII—*Concluded*

No. of rabbit	Substance tested	Dose	Diluting menstruum	Results
Fatty acid V and its major fraction, phthioic acid— <i>Concluded</i>				
R 921	Fatty acid V	12 of 20 mg.	0.5 cc. mineral oil	Omentum markedly thickened; serosal cells filled with refractile bodies; scattered epithelioid cells, in division, striking reaction; some epithelioid giant cells; few free leucocytes; plasma cells
R 982	Phthioic acid	12 of 5 mg.		Omentum markedly thickened; clasmacytes containing leucocytes or yellow debris; no peritonitis; monocytes predominating reaction with a few epithelioid cells; great increase in mononuclear free cells; many plasma cells
R 983				Massive thickening of omentum, weight 18.5+ gm.; many scattered epithelioid cells in active division; epithelioid giant cells; few leucocytes; plasma cells

With such a diffuse and slight reaction it is difficult to judge whether the increase was more than occurs with the mineral oil alone, but our estimate is that there was a slight specific reaction beyond that of the mineral oil. Both omenta showed some scattered leucocytes, some plasma cells, a marked formation of new blood vessels, and in the case of Rabbit R 919 much increase in non-specific connective tissue.

Rabbits R 980 and R 981 which received twelve doses of 5 mg. of the optically inactive tuberculo-stearic acid, showed a less marked reaction than the two rabbits just described, and markedly less than the two animals with corresponding doses of the optically active substance (R 982 and R 983). Rabbits R 980 and R 981 had only a moderately thickened omentum. Again the giant cells, which were largely of the non-rosette type, predominated over epithelioid cells. Epithelioid cells were in minimal numbers, estimated as not more than in the mineral oil controls. In Rabbit R 980 there was one structure which under low power looked like a tubercle, but under the oil immersion lens there were only a few typical epithelioid cells in it; the majority of the cells were highly vacuolated forms, with practically no dense cytoplasm. This structure was partly surrounded by lymphocytes. Thus we estimate that tuberculo-stearic acid has almost no specific

power in the production of epithelioid cells and only a little tendency toward the production of non-specific connective tissue, a finding in conformity with the reaction to commercial stearic acid itself. All four animals in this group showed plasma cells in considerable numbers. The fact that the dosage of the purified form was only one-fourth that of the fatty acid IV makes these conclusions subject to revision, if dosage subsequently proves to be a major factor.

Four rabbits (Table VII) received the optically active forms of the fatty acid from the glycerides. Rabbit R 920 (fatty acid V) proved to be unsatisfactory for the experiment on account of the great excess of adipose tissue in the peritoneal cavity. The animal died 2 days after the ninth injection, and the cause of death could not be determined at autopsy, which was made immediately after death. The omentum showed some stimulated areas in which the cells, as seen supravivally, were of the size of epithelioid cells, but were wholly filled with refractile bodies which in sections appeared as vacuoles. Their nature was not determined. The other three rabbits, R 921, R 982, and R 983, all showed a massively thickened omentum. The first one, R 921, received the fatty acid V (20 mg.) before the purification and the other two the smaller doses (5 mg.) of the phthioic acid. In Rabbit R 921 scattered epithelioid cells were a striking reaction with marked cell division, as is shown in Fig. 27. It will be noted in this section that there is practically no tendency toward the formation of tubercle-like clumps. Supravivally the milk spots of the omentum were seen to be filled with epithelioid cells. There were giant cells, many of them of the rosette type. There was also much non-specific connective tissue, with leucocytes, lymphocytes, and plasma cells. With the purified phthioic acid, the two animals differed in the specific response. In Rabbit R 982 the specific reaction was predominantly of monocytes, as seen in the fresh tissue, with only occasional epithelioid cells; in Rabbit R 983 there were more epithelioid cells, nearly as many as in Rabbit R 921. These cells were also in active division, so that Fig. 27 is a fair representation of them also. The comparison of the tissues of R 921 and R 983 is difficult because there was so much greater increase in the non-specific connective tissue in Rabbit R 983 than in R 921, which makes the epithelioid cells in R 983 appear more diffuse. If they are estimated as approximately the same in amount, the purified form of the fatty acid would be considered the more potent, since the dose was one-fourth as large.

Both Rabbits R 982 and R 983 had a marked increase in free, round mononuclear cells, as distinguished from the fixed connective tissue cells. In this regard the tissue is to be contrasted with that from the reaction to the purified wax and its unsaponifiable derivative, with which the non-specific connective tissue was composed more of fixed than of wandering cells. The free mononuclear cells in R 982 and R 983 were of varied types, lymphocytes, monocytes, and plasma cells. Leucocytes in considerable numbers were diffusely scattered in both omenta.

In Rabbit R 983 there was a very great increase in non-specific connective tissue; after the supravital preparations had been made, the omentum weighed 18.5 gm., as contrasted with the weight of the omentum in Rabbit R 981, namely

7 gm. We had not weighed the omenta from the rabbits in our series previously and the varying amount of adipose tissue would make the weights in normal controls subject to a wide variation. But in a few controls which had been given relatively inert substances and had relatively little excess fat, the weights have varied between 1 and 4 gm. In Rabbit R 983 the adipose tissue had been markedly replaced by new connective tissue, so that the former was not a disturbing factor in the weight of 18.5 gm. which may thus be considered as indicating a marked increase in new tissue. In this new tissue the specific reaction is minimal as compared with the non-specific.

In all the animals receiving the derivatives of the glycerides, the diffuse character of the reaction of epithelioid cells stands in contrast to the clumping of epithelioid cells, already described in the case of the phosphatide, and of the waxes and the fatty acids derived from them. Plasma cells have been a constant reaction to all the preparations of fatty acids from the glycerides and have thus been a more consistent response in this group than in any of the other fractions tested. In general, we have not found plasma cells in the acute reactions, those after one, two, and three injections, but they have been present occasionally after the twelve injections.

SUMMARY

The fatty acid I from the phosphatide proved to be too irritating for administration undiluted, but dissolved in a bland oil it produced tubercular tissue in approximately the same amount as the phosphatide and in tubercle-like masses that closely resembled the reaction of the actual infection. Its amount is indicated as + + + +. The chloroform-soluble lipoids insoluble in acetone, which were in the form of wax-like substances, and the liquid saturated fatty acids derived from them caused the production of abundant epithelioid cells and epithelioid giant cells, in the form of tubercle-like bodies. The specific activity of the soft wax and fatty acid III might be estimated as + + +; of the purified wax and fatty acid II as + +. The soft wax was more irritating than the purified wax and caused much more congestion; the purified wax and the unsaponifiable partition from it gave a marked increase in general connective tissue cells.

The original glyceride fraction has not been tested; it contained two fatty acids (IV and V), in much higher proportion (40 per cent) than the phosphatide A-3 and the waxes. Each of these fatty acids was

found to be a mixture of two saturated fatty acids; one, optically inactive, an isomer of stearic acid, and therefore called tuberculo-stearic acid, was relatively inert in the tissues; the other, optically active, and named phthioic acid, caused a production of scattered epithelioid cells but not in aggregates like tubercles. It is probable that the saturated fatty acids from the phosphatide A-3 and from the waxes also contain tuberculo-stearic and phthioic acids in varying proportions.

NON-SPECIFIC REACTIONS TO LIPOIDS, AND CONTROLS

The partitions of the lipid which have proved inert as regards specific ability to produce epithelioid cells are considered in this section, as are also certain other substances used either as diluting menstrua for the fatty acids, or as general controls.

For the study of the direct effect of any substance upon the connective tissues, the intraperitoneal route has certain advantages owing to the nature of the omentum.

This broad, thin membrane affords a most favorable place for the study of the cells comprising the diffuse connective tissues, inasmuch as any substance injected into the peritoneal cavity passes directly to it and affects its elements. From the experimental standpoint, the omentum may be thought of as offering an opportunity to study tissue reactions occurring *in vivo* in a manner somewhat analogous to the method of *in vitro* tissue culture. It does not offer, of course, the opportunity to observe cells under conditions of strict isolation (tissue culture) over a period of days, nor that of experimenting on such strains *in vitro*. On the other hand, it does give opportunity to analyze reactions in a relatively simple background, while the cells are at the same time subject to the factors of general metabolism and physiological environment of the organism as a whole. The analogy to tissue culture rests, of course, on the nature of the milk spots, which are small clumps of young cells that, in the early response to certain stimuli, grow as an almost single strain of cells and radiate out from the edges of the central mass into the membrane so as to simulate the appearances of a culture. Beside this striking reaction within the milk spots, the more general response of the omentum involves an outpouring of cells from blood to tissues, changes in the blood vessels themselves, and changes in the general connective tissue cells of the organ. These cellular activities may be in the nature of increased division, or increased phagocytic action of mature cells, or of maturation of new forms from primitive types; or the formation of cell types which may be considered as pathological. Any specific reaction must be analyzed in terms of all these factors.

Normal Omentum and Peritoneal Fluid.—A study of the normal omentum and peritoneal fluid was a necessary background for our experimental work. There have been three recent surveys of the omentum and its cells (von Möllendorff, 20, Cunningham, 21, and Maximow, 22). For the purposes of the studies here reported, it has been necessary to establish the normal under the same conditions and with the same technique used in testing the fractions from the tubercle bacillus. This has consisted in a supravital study of the living cells of the omentum and peritoneal fluid stained in neutral red and Janus green, the same technique used by Cunningham, correlated with a study of fixed sections and fixed films of the cells.

As is well known, the normal omentum is a thin, double layer of delicate connective tissue in which may be seen a few sparsely scattered, small, opaque areas, the milk spots. When the two layers are carefully separated and one thickness is spread on a slide, it appears to be an intact membrane with complete covering of serosal cells. With the slightest irritation the serosal cells develop refractile bodies and tend to drop off, leaving denuded areas. Mounted on a film of neutral red, the serosal cells show no reaction to the dye and the fibroblasts but little reaction; in each field there are a few scattered, elongated clasmatocytes or macrophages which contain scattered, small masses of debris staining a deep red in the dye. These cells comprise the principal stained elements of the interspaces.

The milk spots are small clumps of cells, sometimes not more than 20 in number. In eleven animals in which the omentum was, as far as could be determined, entirely normal, two types of cells predominated in the milk spots. One of these was the cell we have called the monocyte with typical rosette of neutral red bodies, and the other a cell of like size with neutral red bodies like those of the rosette of the monocyte, but so scattered in the cytoplasm that they do not accentuate the area of the centrosphere. A given milk spot may be entirely of rosette cells, or of cells with scattered neutral red bodies, or mixed. In one rabbit (R 651), the milk spots were densely cellular and made up of round types the size of monocytes or larger, with a dense, uniform cytoplasm that showed scarcely any reaction to neutral red. The nuclei of these cells were large, round, centrally placed, and had conspicuous nucleoli. These cells we consider as more primitive than the rosette cell or the type with scattered neutral red bodies, and not to be identified as monocyte, clasmatocyte, or lymphocyte. It is clear that the study of living connective tissue has for its object the detection of signs of immaturity and differences in function or in functional states within the group of connective tissue cells. Nagel (23) has recently found that the fibroblasts in tissue culture show but little reaction to neutral red in young (24 hour) cultures, while the cells of the more mature cultures stain well.

NON-SPECIFIC REACTIONS TO LIPOIDS

TABLE VIII
Differential Counts of Cells in Peritoneal Fluids

Substance injected.....	None		Distilled H ₂ O	Normal saline	Mineral oil	Palmitic acid in mineral oil	Stearic acid in mineral oil	Sodium glycerophosphate in H ₂ O	Glycerol in mineral oil	Red phosphorus in saline	Polysaccharide	Protein 304-F
Number of rabbits used.....	11		1	1	4	2	3	2	2	1	2	1
Number of injections.....	None		12	12	12	12	12	7 and 12	2	12	12	12
Total cells per c.mm.	Average	Range	12,700	25,200				Few				
Neutrophilic leucocytes.....	2,285	175 to 8,000										
Lymphocytes.....	1.5%	0-3%	2%	3%	25%	7%	13%	13%	32%	44%	51%	70.5%
Monocytes.....	18	2-56	26	16	22	3	10	7	4	0	7.5	3
Clasmatocytes.....	60	10-87.5	63	73	46	54	72	65	60	39	33	5.5
Serosal cells.....	9.5	5-36	9	6	3	5	3	9	1	17	3.5	20.5
Unclassified.....	6	0-31.5	0	1	1	20	2	1	1	0	5	0.5
	5	0-26*	0	0	2	0	0	5	1	0	0	0

* Range 0 to 7 except for Rabbit R 651 with 26 per cent primitive cells.

† These cells (R 640) were of medium size and filled with refractile bodies.

Three of the eleven animals showed small foci of lymphocytes in the milk spots. Beside these strains of cells, an occasional milk spot contains a few long, branched clasmotocytes like those of the interspaces or a round phagocytic cell with the varied vacuoles characteristic of the active clasmotocyte; but in general the milk spots are made of cells that we consider as relatively young, for they are the first to respond with active cell division under experimental stimuli.

The cells of the peritoneal fluid were studied in the same eleven animals with total counts and a differential count made of the living cells. The results are averaged in the second and third columns of Table VIII. These figures are to be compared with the records of all the animals in this series in which counts of the cells of the peritoneal exudate were made.

In these counts the total free cells were below 2,970 per c.mm. in all but one rabbit, the range being from 175 to 8,000. It will be noted that the predominating form was the cell with the rosette, the so-called monocyte, while the more actively phagocytic forms containing irregularly staining débris which were recorded as clasmotocytes averaged 9 per cent. Only one basophil, no eosinophils, and only one monocyte with accentuated rosette were observed. Very few red blood cells were found—0 to 20 while counting 200 white cells.

In one rabbit (R 651) 26 per cent of undifferentiated cells were encountered, which corresponded with a predominance of the same type of cell in the milk spots of the omentum just described. Three of the animals showed definite foci of lymphocytes in the omentum, and in the respective peritoneal fluids these cells made up 34 (R 578), 48 (R 650), and 56 (R 879) per cent of the free cells found.

From these studies it may be accepted that there is so close a correlation between the omentum and the cells free in the peritoneal cavity as to suggest the omentum as the source of the free cells; that the milk spots are collections of young cells, more or less differentiated; that the phagocytic cell of the interspace is the clasmotocyte (macrophage or histiocyte); that the serosal cells and fibroblasts are inconspicuous in supravital films; and that the total number of free cells in the peritoneum is small. Gardner (24) has recently published figures on the normal differential count of the cells of the peritoneal fluid in guinea pigs, and his average and limits of variation for the different types closely parallel those here cited for the rabbit.

Effects of the Vehicles Used in Administering the Tuberculo-Lipoid

Water and Normal Saline.—In general two forms of diluting menstrua were obligatory in the fraction-testing experiments, aqueous for the phosphatide and a bland oil for the fatty acids and waxes. It

was obviously necessary to determine the effects of the menstrua as such.

There is an outpouring of neutrophilic leucocytes within 5 minutes after the injection of any fluid into the peritoneal cavity, a fact that has recently been emphasized again by Gardner. It has been necessary, therefore, to determine the state of the omentum after a series of injections of distilled water or of physiological saline, the diluents of choice for the introduction of any substance into the peritoneal cavity. In every instance the water employed in these experiments was freshly distilled from glass and sterilized immediately before using. From Table VIII it will be seen that the differential count of the free cells after injections of distilled water remained practically unchanged. Though the total cells per c.mm. showed a decided increase over the normal there was no percentage increase in neutrophilic leucocytes. In the animal (R 709) receiving the distilled water, the parietal and visceral peritoneum was entirely normal in appearance, except for a slight increase in vascular dilatation in the omentum. Microscopically there was evidence of a slight reaction, some of the serosal cells being rounded up with a few refractile droplets and the clasmotocytes with stained debris being slightly increased in number in the interspaces. The milk spots consisted, as normally, of monocytes and clasmotocytes, with an occasional monocyte having an increased rosette; no giant cells were seen. There were foci of both primitive cells and lymphocytes. No abnormal findings were recorded in any of the organs except in the bone marrow which showed supravitaly a decrease in the usual fat content and on section revealed shrunken fat cells in a granular matrix. The myeloid and erythroid cells were entirely normal in their relationships. No anemia and no leucocytosis in the peripheral blood developed during the period of the injections.

In Rabbit R 730 the twelve daily injections of freshly prepared normal saline again increased the total number of free cells found in the peritoneal fluid without altering appreciably their normal percentages (Table VIII); the omentum showed no changes in the gross, and, supravitaly, showed essentially the features mentioned as present after distilled water. In this animal, however, there was a sudden drop of 1,500,000 in the number of red blood cells per c.mm. and of 15 per cent in the hemoglobin (Newcomer) in the last 3 days of the injection period. At autopsy a local peritonitis at the site of injection over the wall of the cecum was encountered; this may have been responsible for the anemia. At no time was there found any increase of total white blood cells. The bone marrow was gelatinous and there was decreased fat content with a shift to earlier developmental forms in both red and white cell series.

It seems evident, then, that either the immediate increase of leucocytes in the peritoneal cavity initiated by the injection of salt solution or distilled water into the peritoneal cavity is transitory or, with repeated injections the response becomes minimal, because 48 hours

after the twelfth injection, neither peritoneal fluid nor omentum showed any increase over the normal in number of neutrophilic leucocytes. The fixed sections showed only scattered plasma cells and a few clumps of monocytes with an occasional epithelioid cell and clasmatocyte.

As has been explained in a preceding section (page 28), the specific, liquid, saturated fatty acids could not be given in water, and when given undiluted were so corrosive that any specific reaction was largely masked by an extreme irritation. Moreover, the waxes and their derivatives were not miscible with water, so an effort was made to find a bland oil as a menstruum. Olive oil, oleic acid, and two purified mineral oils were successively tested for their effect on the peritoneum in the hope that a relatively inert diluent might be found.

Olive Oil.—Five rabbits (R 338, R 341, R 665, R 800, and R 829) received olive oil intraperitoneally in daily doses of 5 cc. each. The severity and extent of the reaction varied somewhat in the several animals but even from the least reactive it was apparent that the irritative effect of olive oil was so great that it would mask effectively any specific reaction that might be expected from a substance given in combination with it. The effects observed ranged from a moderate omental reaction, consisting chiefly of many foreign body giant cells scattered singly and with little diffuse cellular reaction otherwise (R 341), through massive thickening with foreign body giant cells, clasmatocytes, and fibroblasts predominating (R 338), to a massive reaction involving monocytes, epithelioids, and epithelioid giant cells and foreign body giant cells in great abundance (R 829). In the last instance, in which trypan blue was given intravenously together with olive oil intraperitoneally, while there were areas suggestive of tubercular granulation tissue, the tissue in general did not show this reaction to the degree characteristic of the phosphatide A-3 and of the tuberculo-fatty acids. See protocols, page 57.

Oleic Acid.—Oleic acid was identified as one of the constituent fractions composing the original phosphatide A-3 of the tubercle bacillus and was considered as a possible menstruum for the introduction of other fractions. Six rabbits were given various dosages of the oleic acid. In all these there was a marked corrosive action on the peritoneal tissues. The smallest amount given—0.1 cc. in a single injection—was followed by a leucocyte-clasmatocyte reaction with abscess formation within 24 hours. Larger amounts over longer periods gave an accentuation of this reaction with foreign body giant cells and a marked non-specific irritative response.

It is thus clear that oleic acid plays only a non-specific rôle in tuberculosis if it is liberated in the tissues in the process of bacillary dis-

integration, but that inherent irritative properties eliminate it as a possible menstruum for other substances.

Mineral Oil.—A relatively pure mineral oil (Nujol) was next injected in a series of ten rabbits at different times and in different dosages, and, while again the reaction varied in different animals, it was found in general to be the least irritative of any of the solvents for lipoids tested. After from one to three injections of 0.5 cc. each the reaction was negligible. Three only of the eight rabbits receiving from ten to twelve injections of the mineral oil showed any considerable evidence of reaction at autopsy. All had appreciable quantities of the oil still free in the peritoneal cavity but in only one animal (R 663) was there any evidence of irritation other than in the omentum. In general the more striking effect was a non-specific stimulation of connective tissue involving clasmatoocytes and fibroblasts. The nature of this reaction is shown in Fig. 19, Plate 6, which is a spread of the omentum from Rabbit R 661, stained with neutral red and photographed while the cells were still living. It shows a general increase in fibroblasts and in number and activity of elongated clasmatoocytes of rather uniform type. This area is representative of the thinner portions of the omentum from this rabbit. In the thicker parts of the tissue the reaction was less uniform and showed granulocytes scattered diffusely, and a considerable number of foreign body giant cells. There was some monocytic response and a minimal reaction of epithelioid cells.

The minimal reaction observed after the use of this mineral oil alone established its value as a menstruum for introducing substances insoluble in water.

A still further purified, less viscous mineral oil (No. 4 Socony white oil, Standard Oil Company of New York), when given in one animal in the same repeated dosage as the Nujol, showed a reaction the equivalent of the least irritative response found after the latter, and can also be used as a diluent.

Special Controls for the Phosphatide Reactions

Killed Tubercle Bacilli.—The first reaction analyzed as an essential control was, of course, that elicited by the original killed tubercle bacilli themselves.

That dead tubercle bacilli produce tubercular tissue has long been known; the historical significance of this reaction has been traced in a previous report (3). The matter was conclusively settled in 1891 by Prudden and Hodenpyl (18, 19), but in their experiments injections of "2 to 3 cc. of a milky suspension" were used, from which the dose cannot be estimated, the experiments having been done before methods of weighing and counting of bacilli in suspensions had been em-

ployed. In the original phosphatide A-3, there was a small amount of material which was acid-fast and our first control was to inject a small dose of killed bacilli of the strain under investigation, H-37, in quantities (a total of from 3 to 7 mg. in fourteen doses) estimated to approximate the amount of acid-fast debris in a standard dosage of the phosphatide. The results of our experiments were reported in detail in 1927 (3). Small and infrequent tubercles were found proportional to the small dose of organisms but in no instance was there the quantitative reaction observed after the administration of phosphatide. As a further control, it was thought of interest to determine if possible the effect of heat-killed bacilli in dosage (1.6 gm.) the equivalent of the 80 mg. of the phosphatide A-3. In this dosage the phosphatide has been given in 10 cc. distilled water in twelve daily injections without any symptoms whatever. A single corresponding dose of 1.6 gm. of dead tubercle bacilli, H-37, killed each of two rabbits (R 1084 and R 1085) in less than 24 hours. The bacilli were found massed in the peritoneal cavity with relatively little absorption, the fatalities being due evidently to some soluble toxic products accompanying this large dosage.

Lecithin.—When it was found that the phosphatide A-3 gave such an extensive formation of epithelioid cells, another phospholipin, lecithin, prepared from brain tissue by Dr. P. A. Levene, to whom we are indebted for the material, was tested for comparison.

Two rabbits, R 243 and R 244, each received to begin with fourteen intraperitoneal injections of 80 mg. and then one of 122 mg. of lecithin each in 10 cc. distilled water. Though the amount was thus greater than that of the phosphatide A-3 from the tubercle bacillus, the cellular reaction was much less. In R 243 the omentum was almost normal in thickness in the gross, except that at one end there was a pink nodule which looked, with the unaided eye, like the reaction to phosphatide A-3. There were also one or two nodules in the mesentery. In R 244 much of the omentum was thin, but there were some pink bands too thick to study supravitaly. The reaction was complex; there were many of the large, vacuolated cells, such as we have found after one, two, and three injections of the phosphatide A-3 (Figs. 1-3); but when these cells, which had stained brilliantly in neutral red, were faded in formalin and then restained with Sudan III, they gave the typical reaction of fat. In sections the reaction of the less dense areas might be described as a polymorphonuclear and round cell infiltration; there were clasmatoocytes loaded with debris and with leucocytes, many free leucocytes, lymphocytes, and plasma cells, together with large, round, highly vacuolated cells, and a few scattered epithelioid cells. The nodules, on the other hand, which were seen as pink tissue in the fresh, proved to be typical, tubercle-like clumps of epithelioid cells with a few Langhans' giant cells; some of these foci were surrounded with lymphocytes and a few had been invaded with leucocytes.

The lecithin gave a mixed reaction with some production of tubercular tissue, typical in appearance, but minimal in amount, as compared with the reaction to the phosphatide A-3.

As a test of the biological reaction to *phosphorus*, one animal was given red phosphorus in normal salt solution with a marked granulocytic response, and some apparent formation of new young monocytes. Lawrence and Huffman (25) have observed an increase in the number of monocytes in the blood following six to seven subcutaneous injections of yellow phosphorus in oil.

Non-Specific Lipoid Fractions

The phosphatide A-3, Table I, yielded, upon analysis, four subfractions which did not produce epithelioid cells, and a fifth, a sugar acid which has not been tested. The non-specific irritant action of oleic acid has been described in a preceding section of this chapter, which dealt with the search for a bland menstruum in which to administer more corrosive substances.

Palmitic Acid.—Four rabbits (see protocols appended) received palmitic acid in mineral oil in amounts equivalent to those of the saturated fatty acids. There was in each instance an irritative reaction, entirely non-specific, involving primarily clasmatoocytes.

Glycerophosphoric Acid.—The glycerophosphoric acid secured from the phosphatide was given as sodium glycerophosphate in distilled water in amounts equivalent to those which were received by the animals given 80 mg. of phosphatide per dose. The only tissue reaction was a minimal response of leucocytes and clasmatoocytes. Likewise, when glycerol C.P. was given in mineral oil, only the finding of phagocytized oil droplets in the cells of otherwise unstimulated milk spots gave evidence of the series of injections the rabbits had received.

Glucose and the *sugar acid* have not been tested on the cells of the omentum. Thus, the biological evidence for the non-specificity of the subfractions, other than the saturated fatty acid, from the phosphatide, confirms the original view that the principle active in the production of tubercular tissue is quantitatively contained in the fatty acid.

Stearic Acid.—An isomer of stearic acid, which Dr. Anderson has termed "tuberculo-stearic acid," was isolated as one of the constituents of the fatty acid derived from the glyceride fraction. The non-

specific character of the tissue response to this acid has been described in detail in the preceding part of this communication. For comparison stearic acid C.P. was given to five rabbits (see protocols appended). There resulted a minimal reaction, even less than that elicited by its isomer; it reflected a mild response of clasmatoocytes, monocytes, and epithelioid cells.

The maximum response of non-specific diffuse connective tissue was elicited by the unsaponifiable partition from the purified wax. The details of this reaction have been included in the preceding section.

A comparative study of the substances reported in this chapter which have not produced epithelioid cells, reveals three as outstanding from the rest in their reactions in the tissues: lecithin, olive oil, and the unsaponifiable substance from the purified wax. Lecithin is the only substance used as a control thus far which has given tissue that could be called tubercular or tubercle-like in its structure. This particular reaction was, however, minimal in amount when compared with the reaction to the phosphatide A-3 from the tubercle bacillus, and consisted of a few localized tubercles in omentum, mesentery, and wall of the intestine in areas otherwise not much thickened. The olive oil gave rise to an extreme reaction of large, foreign body giant cells. The unsaponifiable substance produced fully as much new tissue as the phosphatide A-3, but none of it was in the form of tubercles; instead there was a marked new growth of general connective tissue cells. These three reactions were sufficiently distinctive for the nature of each irritant to be identified from a study of the tissues.

In all the other controls, the calling of leucocytes into the tissues and a stimulation of clasmatoocytes to phagocytic action have been the common reactions; there have also been some increase in monocytes, a minimal formation of epithelioid cells, the production of giant cells, plasma cells, and, occasionally, a local increase in lymphocytes. None of these reactions, however, have been sufficiently distinctive to enable one to identify from the appearance of the sections the reagent used.

PROTOCOLS OF RABBITS

Controls. All Injections Intraperitoneal

R 709. 12 doses of 10 cc. aq. dest.—No increase in milk spots, slight vascular dilatation in omentum. Clasmatoocytes and serosal cells predominant. No in-

crease of monocytes. Foci of primitive cells and lymphocytes. Bone marrow showed decrease in fat content.

R 730. 12 doses of 10 cc. normal saline.—Omentum showed no change in the gross. Supravitality, findings similar to those in R 709.

R 247. 12 doses of 0.25 mg. H-37 tubercle bacilli (inactivated at 60°C. for 1 hr.) in 10 cc. normal saline. Total of 3 mg. in 14 days.—Omentum showed some increase in milk spots with clasmatoocytes and lymphocytes predominating. Very few epithelioid and Langhans' giant cells seen. One small typical tubercle found in fixed tissue.

R 248. 16 doses of from 0.25 to 3 mg. H-37 tubercle bacilli (inactivated at 60°C. for 1 hr.) in 10 cc. normal saline. Total of 7 mg. in 19 days.—Omentum definitely stimulated. Many clasmatoocytes, plasma cells, and a marked infiltration of lymphocytes. Occasional area of beginning necrosis with leucocytic infiltration. Tubercles were small and all surrounded by a dense thick wall of lymphocytes. Typical tuberculous granulation tissue in limited amount.

R 243. 15 doses of from 80 to 122 mg. lecithin in 10 cc. aq. dest. Total of 1,242 mg. in 17 days.—Omentum appeared normal in the gross except for one pink nodule and some vascular dilatation. Increased phagocytosis by clasmatoocytes. Reaction of perivascular adventitial cells. The nodule was a clump of epithelioid cells like a tubercle with a few Langhans' giant cells.

R 244. 15 doses as in R 243.—Omentum showed moderate thickening with some pink bands. Predominant reaction was of clasmatoocytes highly phagocytic, with many large vacuoles. Phagocytized material all stained with neutral red and afterward with Sudan III. Occasional epithelioid cell seen in supravital films and the pink bands were clumps of epithelioid cells with a few epithelioid giant cells.

R 245. 15 doses of lecithin plus killed tubercle bacilli in 10 cc. aq. dest. Total of 1,242 mg. lecithin and 3.75 mg. tubercle bacilli in 17 days.—Omentum maintained its normal delicacy but with decided increase in milk bodies. Supravitality there were many epithelioid cells and clasmatoocytes, with serosal cells prominent.

R 246. 15 doses. Total of 1,242 mg. lecithin and 4 mg. tubercle bacilli in 17 days.—Omentum showed some vascular dilatation, with milk spots only moderately apparent. Predominant reaction was of clasmatoocytes and lymphocytes with occasional epithelioid and Langhans' giant cells.

R 338. 15 doses of 5 cc. olive oil.—Few adhesions and great quantity of fat in peritoneal cavity. Omentum thickened, with many clasmatoocytes, fibroblasts, and very large foreign body giant cells. Epithelioid cells and epithelioid giant cells few and scattered.

R 341. 15 doses of 5 cc. olive oil.—Massive fat in peritoneal cavity. Omentum only moderately stimulated, much of it remaining delicate and thin. All connective tissue cells filled with oil droplets. Very few epithelioid cells. Predominant reaction was of large foreign body giant cells scattered singly in the areolar tissue with very little cellular reaction otherwise. Very few epithelioid giant cells.

R 665. 11 doses of 5 cc. olive oil.—Serosa of cecum markedly irritated; omentum thickened, fibrous, and inflamed. Much fat, free and adherent, and an excess of opaque oily fluid in peritoneal cavity. Predominating cell in fluid and omentum was the monocyte, with many single and multinucleated epithelioid cells. There were more large foreign body giant cells than small epithelioid giant cells. Reaction mixed and very complex. Several small abscesses with infiltration of neutrophils.

R 800. 2 doses of 5 cc. olive oil plus 2 cc. 1 per cent Niagara blue intravenously.—Predominantly a clasmotocytic reaction as proven by accompanying intravital stain. Few neutrophils. Very little reaction in general.

R 829. 12 doses of 5 cc. olive oil plus 1 per cent trypan blue intravenously. Total 26 cc. trypan blue in 13 days.—More general cellular reaction in omentum than in other rabbits with olive oil. Monocytes, epithelioid cells, and small epithelioid giant cells were predominant. Clasmatocytes and foreign body giant cells complicated the picture. Trypan blue in finely divided state formed the rosette in the epithelioid series and appeared in larger aggregations in clasmtoocytes and foreign body giant cells.

R 398. 1 dose of oleic acid 0.1 cc.—Many neutrophilic leucocytes. Some phagocytized by clasmatocytes. A few young monocytes seen. One small abscess. Otherwise normal omentum.

R 400. 2 doses of 0.1 cc. oleic acid.—Adhesions between cecum and peritoneal wall, sharply localized. Omentum normal in the gross. Many neutrophils and clasmatocytes in certain areas.

R 402. 3 doses of 0.1 cc. oleic acid.—Adhesions between cecum and parietal peritoneum. Serosal cells irritated. Most of neutrophils are in clasmatocytes. Young monocytes but no epithelioid cells seen.

R 435. 1 dose of 1 cc. oleic acid.—Excess of peritoneal fluid with white granular exudate over cecum and liver. Omentum showed vascular dilatation and many neutrophils and clasmatocytes. Few young monocytes present. Multiple abscesses.

R 444. 1 dose of 1 cc. oleic acid.—Some increase of milk spots in omentum but otherwise content of peritoneal cavity normal in gross. Many actively phagocytic clasmatocytes and young monocytes. Few changes of primitive cells. No epithelioid cells seen.

R 1025. 12 doses of 0.1 cc. oleic acid in 0.5 cc. mineral oil.—Adhesion of cecum and loop of small intestine to peritoneal wall. Much fibrinous exudate. Omentum moderately thickened. Clasmatocytes the predominant reaction. Neutrophils and fibroblasts, but very few monocytes. No epithelioid cells. Few foreign body giant cells.

R 459. 1 dose of 1 cc. mineral oil.—Omentum normal. Milk spots composed of clasmatocytes. Few young monocytes, and plasma cells. No epithelioid cells. No increase of neutrophils.

R 511. 3 doses of 0.5 cc. mineral oil.—Omentum normal. Branched clasmatocytes not stimulated.

R 503. 12 doses of 0.5 cc. mineral oil daily.—Small amount of opaque peritoneal fluid containing many neutrophils, monocytes, serosal cells, and occasional epithelioid cells and clasmotocytes. Much fat in omentum; otherwise normal. Clasmotocytes contained some phagocytized red and white blood cells. Few monocytes, occasional epithelioid cell. Many neutrophils. Many vacuolated mononuclears. Very occasional vacuolated giant cell. Plasma and primitive cells.

R 621. 12 doses of 0.5 cc. mineral oil daily.—Omentum entirely normal in the gross. Many foci of plasma cells and lymphocytes. Some vacuolated mono- and multinuclear cells. Occasional neutrophils. Some monocytes and few epithelioids, and foreign body giant cells.

R 622. 12 doses of 0.5 cc. mineral oil daily.—Peritoneal cavity normal and very few cells in the opaque fluid. Omentum only showed evidence of reaction. Many monocytes and clasmotocytes, few epithelioid and epithelioid giant cells. Many foreign body giant cells, some with vacuoles. Frequent foci of lymphocytes.

R 660. 10 doses of 0.5 cc. mineral oil daily.—Peritoneal cavity contained a small amount of opaque fluid. Cell count 7,000 per c.mm. Neutrophils 13 per cent; lymphocytes 19 per cent; monocytes 66 per cent; clasmotocytes 1 per cent; unclassified 1 per cent. Omentum showed stimulation only in the portion dorsal to the greater curvature of the stomach. Areas between milk spots practically unstimulated. Many of cells of milk spots contained oil droplets obscuring cytoplasmic structures. Typical monocytes and clasmotocytes abundant. No neutrophils or lymphocytes. Epithelioid giant cells and foreign body giant cells very rare.

R 661. 12 doses of 0.5 cc. mineral oil daily.—Small amount of opaque fluid in the peritoneal cavity contained 26,800 cells per c.mm. Neutrophils 21 per cent; lymphocytes 30 per cent; monocytes 33 per cent; clasmotocytes 6 per cent; unclassified 10 per cent. Omentum showed some increase of milk spots and a few pink, thickened areas. Some plasma cells, monocytes, occasional epithelioid cell and epithelioid giant cell. Many lymphocytes. Predominant reaction was of clasmotocytes, fibroblasts, and foreign body giant cells. See Plate 6, Fig. 19.

R 662. 12 doses of 0.5 cc. mineral oil every 48 hours.—Some increase of fluid in the peritoneal cavity. Neutrophils 36 per cent; lymphocytes 22 per cent; monocytes 39 per cent; clasmotocytes 2 per cent; serosal cells 1 per cent. Omentum showed only a moderate increase of milk spots, composed either of clasmotocytes or monocytes. Interspaces unstimulated. Both epithelioid and foreign body giant cells few in number. Occasional epithelioid cell, monocytes frequent.

R 663. 12 doses of 0.5 cc. mineral oil every 48 hours.—Cellular peritoneal fluid. Neutrophils 30 per cent; lymphocytes 19 per cent; monocytes 46 per cent; clasmotocytes 2 per cent; serosal cells 2 per cent. Considerable white exudate, and omentum thickened. Branched stimulated clasmotocytes. Small foci of monocytes with some multinucleated rosette cells. Also foreign body giant cells. Many scattered neutrophils and occasional abscesses.

R 1030. 12 doses of 0.5 cc. mineral oil.—Considerable free oil in peritoneal cavity. Omentum showed a very slight reaction of milk spots. Clasmotocytes with phagocytized neutrophils predominated. Some milk spots were composed of monocytes. Both eosinophils and neutrophils present.

R 1060. 12 doses of 0.5 cc. H-4 purified mineral oil.—Small amount of free opaque oily fluid in peritoneal cavity. Omentum showed some increase in milk spots, with undifferentiated cells and some monocytes. Very rare giant cell of either type found. Predominating cell, clasmatocyte. Minimal reaction on whole.

R 554. 2 doses of 16 mg. palmitic acid in 0.5 cc. mineral oil.—Most of cells of omentum unstimulated. Few neutrophils, clasmatocytes with phagocytized white blood cells. Usual monocytes.

R 555. 3 doses of 16 mg. palmitic acid in 0.5 cc. mineral oil.—Some irritation of serosa of cecum. Omentum delicate but with increased milk spots, made up chiefly of young monocytes. Occasional epithelioid cell. Very little stimulation otherwise.

R 1026. 12 doses of 16 mg. palmitic acid in 0.5 cc. mineral oil.—Fibrinous exudate on cecum. Omentum thickened; increased vascularity with general clasmatocyte stimulation. Few monocytes.

R 1028. 12 doses of 16 mg. palmitic acid in 0.5 cc. mineral oil.—Cecum covered with fibrinous exudate, also diaphragm. Omentum thickened with overwhelming clasmatocytic reaction. Few monocytes and lymphocytes. Some indifferent connective tissue cells.

R 553. 2 doses of 16 mg. stearic acid in 0.5 cc. mineral oil.—Omentum and peritoneal cavity entirely normal in gross and under the microscope.

R 559. 3 doses of 16 mg. stearic acid in 0.5 cc. mineral oil.—Omentum, otherwise normal, showed prominent milk spots, made up of clasmatocytes with leucocytes phagocytized. Few foci of monocytes. Interspaces entirely unstimulated.

R 1057. 12 doses of 5 mg. stearic acid in 0.5 cc. mineral oil.—Only a very slight reaction in peritoneal cavity. Omentum contained very small milk spots, composed of monocytes. Clasmatocytes branched and unstimulated in interspaces. Occasional epithelioid cell, rarely with multiple nuclei.

R 1058. 12 doses of 5 mg. stearic acid in 0.5 cc. mineral oil.—Some free fibrinous plaques in peritoneal cavity. Omentum showed diffuse thickening. Few neutrophils and lymphocytes. Many young monocytes, few epithelioids, occasional epithelioid giant cell. Some increase in fibroblasts and clasmatocytes.

R 1059. 12 doses of 20 mg. stearic acid in 0.5 cc. mineral oil.—Adhesion between cecum and parietal peritoneum. Very little evidence of reaction in omentum. Free and phagocytized neutrophils in clasmatocytes. Dominant cell was the monocyte, with occasional epithelioid cell and epithelioid giant cell. Some large foreign body giant cells.

R 638. 2 doses of 16 mg. glycerol in 0.5 cc. mineral oil.—Omentum entirely unstimulated in gross, and microscopically the usual clasmatocytes with few monocytes were found.

R 639. 3 doses of 16 mg. glycerol in 0.5 cc. mineral oil.—Omentum had accentuated milk spots, but the interspaces were entirely normal. Clasmatocytes were in the interspaces and comprised some of the milk spots; monocytes were found in others; clasmatocytes contained oil droplets.

R 640. 12 doses of 16 mg. glycerol in 0.5 cc. mineral oil.—Omentum showed no gross changes. Milk spots contained both monocytes and undifferentiated cells. All the oil droplets were limited to the cells of the milk spots. Clasmatocytes remained unstimulated in the interspaces. Few plasma cells. One foreign body giant cell seen with large vacuoles.

R 832. 7 doses of 4 mg. sodium glycerophosphate (from H-37 A-3) in 10 cc. aq. dest.—Peritoneal cavity and viscera normal. Omentum normal in the gross, showed microscopically only increased neutrophils, both within and without clasmatocytes. Usual monocytes. No giant cells.

R 833. 12 doses of 4 mg. sodium glycerophosphate (from H-37 A-3) in 10 cc. aq. dest.—Very slight increase in number and size of milk spots in the omentum. No thickening. Milk spots composed of phagocytic clasmatocytes, with considerable number of neutrophils. Few monocytes and small lymphocytes. Essentially normal otherwise.

R 433. 1 dose of 0.1 cc. suspension red phosphorus in 1.9 cc. NaCl.—Peritoneal irritation at site of injection. Scraping showed many monocytes. Increased prominence of milk spots only in omentum. Neutrophils and clasmatocytes in moderate number. Foci of young monocytes prominent; some plasma cells.

GENERAL DISCUSSION

The first significant property of chemical partitions derived from bacterial analyses is solubility. On this basis, the fractions from the tubercle bacillus can be divided into two primary groups, the one water-soluble, the other water-insoluble; the former includes the proteins and polysaccharides, the latter the lipoids. The group of the acid-fast organisms is well known to be especially rich in lipoids. It, therefore, has long been recognized as important to determine the biological significance of the tuberculo-lipoids.

The chemical basis for the studies here reported is to be found in the comprehensive analysis made by Dr. R. J. Anderson (9) of the lipoids isolated from one strain of tubercle bacilli, H-37, grown under standard conditions on Long's synthetic media. He has submitted to us all the primary divisions of these lipoids and many of the sub-fractions. As far as we know, there never before has been the opportunity to test biologically all the different lipoids from one analysis. This chemical study is to be the pattern for the subsequent analyses of

the corresponding material from the other acid-fast organisms, and the biological testing, as here outlined, may thus also serve as a guide for such parallel tissue studies. From the biological standpoint, it has proved of great importance to be able to compare the relative potencies of all the lipid partitions in the production of epithelioid cells, as well as to determine their power to incite general acute and chronic inflammatory reactions in the connective tissues.

The lipoids from the tubercle bacillus, Strain H-37, have been split by Anderson (9) into four groups, of which two, phosphatide and glycerides, are soluble in alcohol-ether, and two, purified wax and soft wax, in chloroform. In a further grouping, the phosphatide, purified wax, and soft wax are insoluble and the glycerides soluble in acetone. These lipoids and split products from them have been administered to rabbits in the following ways: isolated (fatty acid I), as an emulsion in water (the phosphatide), in solution in water (non-specific substances, oleic, palmitic, and glycerophosphoric acids), or in mineral oil (all the saturated fatty acids, I-V, tuberculo-stearic acid, phthioic acid, the waxes, and the unsaponifiable substance).

In general, the reactions of the normal connective tissue of the rabbit to the lipoids from the tubercle bacillus may be divided into three groups: first, the acute irritative response, involving dilatation of the vessels and an emigration of leucocytes with their subsequent ingestion and disintegration by clasmatocytes or with abscess formation; second, the so-called specific development of epithelioid cells and epithelioid giant cells; and third, the no less significant but more general changes in the connective tissues, including an increase in undifferentiated fixed connective tissue cells, fibroblasts, clasmatocytes, young, free, basophilic mononuclear cells, lymphocytes, plasma cells, and fibrous tissue, together with an active new formation of blood vessels.

The Acute Irritative Response.—The calling of leucocytes from the vessels into the tissues is a general initial response to the introduction of almost any foreign substance into the tissues. It is difficult to find a substance which does not call forth leucocytes, and the reaction follows the injection of living or dead tubercle bacilli and all fractions, whether protein, polysaccharide, or lipid, as well as all split products of the lipoids so far tested. In general, the leucocytes thus called out

have in these experiments been phagocytized by clasmatocytes more frequently than aggregated into pus. Certainly when the occasional accidental infections have been ruled out, abscess formation has been a relatively minor reaction.

The most interesting point in connection with the acute irritation caused by these different substances has been the readiness with which the tissues have ceased to respond to succeeding injections of certain of the fractions with a fresh emigration of leucocytes. For example, after the administration of phosphatide, only the first dose has called out any number of leucocytes; by the third injection, only the evidence of their destruction by clasmatocytes remains; with the polysaccharide, on the other hand, each injection brings out a fresh emigration of leucocytes. The undiluted fatty acids have a corrosive action, and repeated injections give a marked, sterile peritonitis. When the acids are dissolved in mineral oil, the irritative effects of repeated doses can be reduced to the constant presence in the tissues of some leucocytes, scattered and in small clumps. The tissues after the administration of fatty acids have never been free from leucocytes, as they are with the phosphatide, and occasionally there have been small abscesses. On the basis of the work of Carrel (26), who found that the white blood cells contain growth-producing substances, the question arises as to whether the new formation of connective tissue following the lipoids is in part due to the destruction of leucocytes with the liberation of such stimuli. In this connection, we may say that the destruction of leucocytes is a factor common to all the experiments and thus can hardly account for any differences in cellular reaction. For example, no portion of the tubercle bacillus calls out more leucocytes than the polysaccharides, and yet these fractions cause a very limited formation of new connective tissue. On the other hand, the phosphatide stimulates a massive formation of new tissue, with only a minor and transient emigration of leucocytes. The experiments with the phosphatide, to which the neutrophils become so quickly non-responsive, make it possible to estimate that the length of time necessary for the complete destruction of leucocytes by clasmatocytes is not more than 2 days.

There is another factor common to the experiments involving repeated doses of the lipoids, namely, that the normal adipose tissue

of the omentum becomes replaced by new connective tissue, so that the possible effects of the breaking up of the lipoids of the tissues themselves must also be taken into account.

Specific Response.—In the production of epithelioid cells and epithelioid giant cells, each of the four primary groups of the lipoids contains certain liquid saturated fatty acids which give a specific reaction comparable in amount to that of the original fraction. Thus it may be said that the four original partitions give a specific reaction proportional to the amount and to the activity of the fatty acids they contain. Arranged in series, the phosphatide and fatty acid I give the maximum reaction, + + + +; the soft wax and fatty acid III, + + +; the purified wax and fatty acid II, + +; and the active fraction from the glycerides, +. The original primary fraction made up of glycerides has not been tested, but in the fatty acids from this partition it has been shown that the specific activity resides in an optically active fraction, phthioic acid, which in fatty acid IV is present in small amount, enough to give some epithelioid cells, while the optically inactive portion of fatty acid IV, separated out and identified as an isomer of stearic acid, is inert toward epithelioid cells. The specific activity of fatty acid V has been shown to be due to its content of phthioic acid. The amount of specific tissue produced, which we estimate as +, is not directly comparable with that found after the other fatty acids, because of the relatively smaller dosage used. Further chemical studies are necessary before it can be known whether phthioic acid is the only active principle for tubercle formation.

The phosphatide, the waxes, and their fatty acids have caused much production of the specific tissue in the form of closely packed masses of epithelioid cells, making tubercle-like structures, while the fatty acids from the glycerides have brought about no clumping of the epithelioid cells. It remains to be seen whether this is simply a matter of dosage or of the amount of the specific material concentrated in a given place.

Giant cells have been a constant factor in these reactions, but the numbers and the types have varied markedly. With the phosphatide, the Langhans type predominated overwhelmingly. Their occurrence varied from one or two giant cells in a mass of epithelioid cells, through all variations in proportion to epithelioid cells up to pure masses of

giant cells. In analyzing the reactions of all other fractions, it must be remembered that the diluting menstruum, mineral oil, gave rise to a few foreign body giant cells when used alone. Giant cells were in greatest numbers with the purified wax and the fatty acid IV; in these two cases, large foreign body types predominated. It has not been possible to determine fully the reason for the occurrence of giant cells, but the supravital studies of the omentum have often revealed an epithelioid giant cell in the midst of a clump of epithelioid cells, in cases in which it was entirely clear that there was no necrosis in the vicinity. The use of the entire intact membrane rather than a section has made this observation possible and has confirmed the view of Doan, Sabin, and Forkner (7) and of Forkner (8) that the Langhans giant cell is simply a multinuclear epithelioid cell and subject to similar stimuli.

Non-Specific Connective Tissue.—Besides these differences in the number of epithelioid cells and giant cells, there are marked differences in the amount of the so-called non-specific tissue reaction. In general this reaction is extremely complex and involves a new growth of blood vessels with active division of endothelium and adventitia, the new formation of undifferentiated cells, of fibroblasts, clasmatocytes, lymphocytes, and plasma cells. In these experiments the formation of fibrous tissue, except in adhesions, has not been extreme and there has been no marked encapsulation of groups of epithelioid cells, even during the process of resorption lasting up to 5 months. The lipoids that produced the most epithelioid cells, namely, the phosphatide and soft wax, produced the least general connective tissue, while the purified wax and the glycerides produced the most. The purified wax and its derivative, the unsaponifiable fraction, caused the development of connective tissue composed largely of the so-called fixed type, while the phthioic acid produced a tissue rich in free, young, mononuclear cells, monocytes, lymphocytes, and plasma cells. An increase in connective tissue is found around tubercles and as a part of tuberculous granulation tissue in the disease itself, and hence it is significant that such a reaction follows injections of the lipoids, indeed in extreme amounts after certain fractions, namely, the purified wax, the unsaponifiable substance, and the glycerides.

The Nature of "Foreign Body Reaction."—The reactions to the lipoids from the tubercle bacillus have been placed by pathologists

in the group of the so-called foreign body reactions. The experimental studies on the phagocytic activity of connective tissue cells initiated by the work of Metchnikoff and involved in the whole history of the study of living cells with reference to their phagocytic power, call for an attempt to formulate a more definite idea of the meaning of the term "foreign body reaction."

The cells of the connective tissues may be subjected to foreign substances in two ways. Either they may become surrounded by materials which are soluble in the fluids which bathe them, for example, proteins and polysaccharides, which in solution would tend to reach all the cells of the body; or, on the other hand, there may be substances introduced into the tissues which are insoluble, or possibly slowly soluble in the fluids which surround the cells. To this latter group belong the lipoids and certain other substances which, owing to their particulate nature, remain unevenly distributed in the tissues as foreign bodies. Beside the fundamental property of solubility, foreign bodies must be classified according to the size of the molecule or particle; and furthermore, according to whether the cells of the tissues are physiologically adapted to deal with them, when they are engulfed. By general agreement, the term phagocytosis means the engulfing of particles of appreciable size, rather than the diffusion into cells of substances in solution. In supravital staining, a dye like neutral red, which enters the cell in solution, or in fine suspension, merely evidences phagocytosis through the property of the phagocytic cells to secrete this dye and similar vital dyes into the vacuoles of segregation, in which phagocytized particulate matter has been placed.

In these studies, there has been the opportunity in each of the four groups of lipoids to study a physiologically active substance, first in the form of a large molecule, and then in the form of that fraction of the molecule that carries the active principle. This can be illustrated in the case of the phosphatide and fatty acid I, inasmuch as with them the amount of the material and the number of experiments have been more adequate. The ultimate effects of injections of phosphatide and fatty acid I have been the same, namely, a massive production of epithelioid cells and epithelioid giant cells, but the immediate effects have been different. Barring the initial irritation, the overwhelming immediate reaction to one, two, and three doses of phosphatide has

been what we interpret as the phagocytosis of the material by the cells of the milk spots. These phagocytes have been large, round cells, filled with large vacuoles of uneven size but stained with a uniform red color in neutral red. They are the vacuolated cells of Figs. 1 to 3 on Plate 1. This same type of reaction follows the injection of lecithin. On the other hand, the reaction to one, two, and three doses of fatty acid I has been the maturation of typical new monocytes from a more primitive stage, with marked cell division, and by the 4th day signs of their transformation into typical epithelioid cells. During the first 3 days of the injections of the phosphatide, the maturation of characteristic monocytes has also taken place, but in relatively smaller numbers, as compared with the phagocytic cells. By the third dose, however, the actual number of cell divisions in the young, non-phagocytic, mononuclear cells is much greater than in the mature, heavily vacuolated forms. The comparison of the early response to the large molecule (phosphatide) and then to the smaller molecule (fatty acid) suggests that in the case of the phosphatide the cells may at first be occupied with a process of chemical disintegration which with the fatty acid has already been accomplished in the test-tube. The simpler substance, the fatty acid, appears to be concerned physiologically with the extremely complex processes included in the term maturation, which involve rapidly repeated cell division during a period in which the cytoplasm changes from an immature to a mature state. There is considerable evidence that the epithelioid cell, with its large rosette of tiny bodies, is derived from the monocyte, with its small rosette of larger bodies; but certain crucial points for the proof of this view are still lacking. We have some means for detecting the general signs of phagocytosis in the vacuoles of segregation, which stain supravitaly, and can identify certain phagocytized materials in these vacuoles, such as carbon, iron, dyes in particulate form, and whole cells, but we have no means of discriminating other chemical waste products and their stages of disintegration. We do not know the nature or content of the vacuoles that make the rosette of the normal monocyte, nor whether this rosette is a preformed organ of the cell or appears only after the cell has begun to function by taking in material. In the monocyte and in the epithelioid cell, the bodies of the rosette react with a uniform color to neutral red, which we assume

indicates that they are at about the same hydrogen ion concentration. It seems clear from these experiments that epithelioid cells can be made to appear by the introduction into the tissues of certain fatty acids and larger molecules from the tubercle bacillus. If this results from the phagocytosis of these substances by monocytes, not by an indirect chemical influence on the cells, then the monocyte and epithelioid cell must have the power of breaking this material into very fine particles, but, after it has been thus finely divided, of changing it only very slowly. In other words, the cell is not physiologically adapted to any such rapid disintegration of this particular phagocytized material as has been demonstrated for the clasmatoocyte, in which whole leucocytes have been reduced to a granular débris in 2 days. Epithelioid cells have been found apparently unchanged 5 months after their production by chemical means. Thus, while it may well be that the formation of epithelioid cells after the lipoids belongs in the general group of so-called foreign body reactions, the processes involved in the reaction are more complex and less well understood than the phagocytosis of materials that can be more readily identified and followed in the macrophages of the tissues.

These studies have shown that in tuberculosis, the production of the essential lesion, the tubercle, or rather of its essential unit, the epithelioid cell, is due to lipoids from the bacillus, and specifically to certain hitherto unknown fatty acids of high molecular weight. It is, of course, well recognized that the epithelioid cell is not the unique reaction of the disease tuberculosis, and hence the demonstration that certain specific lipoids are the effective agents in this disease, important as it is to the pathology of tuberculosis, does not demonstrate that tuberculo-lipoids are the sole agent in the production of epithelioid cells and epithelioid giant cells. In many pathological processes other than tuberculosis, epithelioid cells as such are found; further, they are readily produced, as is shown in our studies with other foreign bodies, especially lecithin, injected into the tissues, and as has been reported in the recent publications of Cunningham and his co-workers (27). It remains to be seen whether there is any common chemical factor in the production of epithelioid cells, or whether the common factor may not be material of varied chemical composition, which the cell can disperse into fine particles, but cannot change or digest with

speed. Because of the differing properties of the phagocytic cells, the power of the monocyte-epithelioid strain to disperse finely yet but slowly change phagocytized material and that of the clasmatocyte or macrophage to digest material quickly, we find convenient the concept that there are two strains of phagocytic cells in the connective tissues differing in their physiological activities. The other alternative is the assumption of a single strain of cells which react differently as the environment is varied.

Beside the opportunity to study the larger and the smaller molecules of a physiologically active substance, the fatty acids from the glycerides have provided an opportunity to study biologically active principles in increasing purity and especially to give an example of an optically active substance which is more biologically active than a similar substance optically inert.

Throughout these experiments, any extreme, acute, inflammatory reaction like that resulting from an accidental infection, or from an extreme reaction to a diluting menstruum, such as olive oil, has tended to obscure the specific formation of tubercular tissue. Caseation has been found only after the injections of the phosphatide.

In spite of great activity in the production of new connective tissue, the lipoids have not induced any skin sensitivity. Intracutaneous skin tests with Old Tuberculin, the protein, lipoid, and polysaccharide fractions have been consistently negative, as well as tests of the conjunctiva in rabbits. Subsequent inoculation with living tubercle bacilli has resulted in no allergic phenomena.

CONCLUSIONS

1. The lipid fractions from the tubercle bacillus contain maturation factors for monocytes, epithelioid cells, and epithelioid giant cells.
2. The most important component of the lipoids for biological investigation is the phosphatide A-3, since this produces the most massive reaction toward epithelioid cells and epithelioid giant cells, and also because it is the only partition of the lipoids which acts as an antigen.
3. The stimulus to the formation of tubercles resides in certain fatty acids of high molecular weight found in tubercle bacilli. These fatty acids are present in the four major partitions of the lipoids and account in each instance for their specific activity. The most potent fatty

acid in the production of tubercles is that derived from the phosphatide. The purified, optically active phthioic acid obtained from the glyceride fraction, in small dosage produces epithelioid cells, but more non-specific connective tissue; the optically inactive tuberculo-stearic acid is relatively inert.

4. The specific tubercular tissue resulting from the intraperitoneal injection of the phosphatide from the tubercle bacillus undergoes resorption. Two mechanisms in its disappearance similar to those operating in the disease have been seen: caseation in which masses of degenerating epithelioid cells become infiltrated with leucocytes; phagocytosis of the cellular débris by clasmatocytes, without caseation.

5. Beside the specific reaction of the lipoids, these also produce a marked growth of non-specific connective tissue cells, without, however, any reaction toward fibrous tissue in the acute stages. All the subfractions from the lipoids are irritating when injected into the peritoneal cavity, calling leucocytes into the tissues and stimulating clasmatocytic activity. The unsaponifiable substance from the purified wax is particularly active in producing an extreme general reaction of connective tissue cells.

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EXPLANATION OF PLATES

PLATE 1

FIG. 1. Section of omentum of Rabbit R 821, after three intraperitoneal injections of 80 mg. of phosphatide A-3 from H-37 in 10 cc. distilled water. The figure shows vacuolated phagocytic cells of a milk spot; one containing an engulfed leucocyte. The two cells with dense cytoplasm on the left border are possibly a monocyte and an epithelioid cell. Hematoxylin and eosin. $\times 1,000$.

FIG. 2. Section of omentum of Rabbit R 821,—to show the range of free cells, vacuolated phagocytic cells, some containing leucocytes, free leucocytes, lymphocytes, and possibly monocytes. Hematoxylin and eosin. $\times 1,000$.

FIG. 3. Section of omentum of Rabbit R 821,—to show cell division of the monocytes. Hematoxylin and eosin. $\times 1,000$.

FIG. 4. Film of living omentum of Rabbit R 664, after eleven intraperitoneal injections of 80 mg. of phosphatide A-3 in 10 cc. distilled water,—to show milk spot consisting of epithelioid cells supravitally stained in neutral red. The material stained red in the living state appears dark in the photograph. Supravital neutral red. $\times 95$.

PLATE 2

FIG. 5. Section of omentum, Rabbit R 478, after seven intraperitoneal injections of 10 mg. of phosphatide A-3 from H-37 in 2 cc. distilled water,—to show a small group of typical epithelioid cells with dense cytoplasm. One in division. Hematoxylin and eosin. $\times 790$.

FIG. 6. Section of wall of cecum of Rabbit R 319, after twelve intraperitoneal injections of 80 mg. of phosphatide A-3 from H-37,—to show a group of atypical epithelioid cells with foamy cytoplasm. Hematoxylin and eosin. $\times 790$.

PLATE 3

FIG. 7. Film of living omentum. Edge of a milk spot of the omentum of Rabbit R 664, after eleven intraperitoneal injections of 80 mg. of phosphatide A-3 in 10 cc. distilled water,—to show the supravital reaction of coarse neutral red granules in epithelioid cells and epithelioid giant cells. Supravital neutral red. $\times 1,050$.

FIG. 8. Film of living omentum of Rabbit R 664, after eleven intraperitoneal injections of 80 mg. of phosphatide A-3,—to show two small epithelioid giant cells with fine neutral red bodies. The refractile bodies represent serosal cells; clasmatoctes are seen indistinctly at a different level. Supravital neutral red. $\times 1,050$.

FIG. 9. Section of omentum of Rabbit R 158, after twelve intraperitoneal injections of 80 mg. of phosphatide A-3 in 10 cc. distilled water,—to show a massive formation of epithelioid cells, with a few epithelioid giant cells and a mass of lymphocytes. Hematoxylin and eosin. $\times 150$.

FIG. 10. Section of omentum of Rabbit R 153, after twelve intraperitoneal injections of 80 mg. of phosphatide A-3 in 10 cc. distilled water,—to show masses of epithelioid cells with many epithelioid giant cells, moderately vascularized. Hematoxylin and eosin. $\times 63$.

PLATE 4

FIG. 11. Section of omentum of Rabbit R 153, after twelve intraperitoneal injections of 80 mg. of phosphatide A-3 in 10 cc. distilled water,—to show small groups of epithelioid cells and tubercular granulation tissue. There are many epithelioid giant cells. Hematoxylin and eosin. $\times 132$.

FIG. 12. Section of omentum of Rabbit R 153, after twelve intraperitoneal injections of 80 mg. of phosphatide A-3 in 10 cc. distilled water,—to show tubercular granulation tissue. Hematoxylin and eosin. $\times 176$.

FIG. 13. Section of a thoracic lymph node along the internal mammary vein of Rabbit R 319, after twelve intraperitoneal injections of 80 mg. of phosphatide A-3 in 10 cc. distilled water,—to show a lesion made wholly of epithelioid giant cells. Hematoxylin and eosin. $\times 397$.

FIG. 14. Section of a thoracic lymph node of Rabbit R 319, after twelve intraperitoneal injections of 80 mg. of phosphatide A-3 in 10 cc. distilled water,—to show a small focus of early caseation. Hematoxylin and eosin. $\times 397$.

PLATE 5

FIG. 15. Section of omentum from Rabbit R 502, which had received twelve intraperitoneal injections of 16 mg. of fatty acid I in 0.5 cc. mineral oil. It shows a tubercle-like structure surrounded by lymphocytes. Hematoxylin and eosin. $\times 195$.

FIG. 16. Section of omentum from Rabbit R 613, which had received twelve intraperitoneal injections of 16 mg. of fatty acid I in 0.5 cc. mineral oil. It shows a multiple, tubercle-like structure with giant cells and a capsule of lymphocytes. Hematoxylin and eosin. $\times 200$.

FIG. 17. Section of omentum from Rabbit R 613. It shows tubercular granulation tissue and both types of giant cells. Hematoxylin and eosin. $\times 200$.

FIG. 18. Section of omentum from Rabbit R 614, which had received twelve intraperitoneal doses of 16 mg. of fatty acid I in 0.5 cc. mineral oil. It shows tubercle-like structures and tubercular granulation tissue. Hematoxylin and eosin. $\times 200$.

PLATE 6

FIG. 19. Film of omentum, supravitality stained in neutral red, from Rabbit R 661, which had received twelve intraperitoneal doses of 0.5 cc. mineral oil. Supravital neutral red. $\times 180$.

FIG. 20. Section of omentum of Rabbit R 615, which had received twelve intraperitoneal injections of 16 mg. of fatty acid II in 0.5 cc. mineral oil. It shows tiny tubercle-like structures, an epithelioid giant cell, and tubercular granulation tissue. Hematoxylin and eosin. $\times 200$.

FIG. 21. Section of omentum of Rabbit R 615. It shows two epithelioid giant cells with a small, tubercle-like mass of monocytes and epithelioid cells between them. Hematoxylin and eosin. $\times 960$.

FIG. 22. Section of omentum of Rabbit R 615,—to show an epithelioid giant cell with central area of necrosis and one phagocytized leucocyte. There are typical monocytes. Hematoxylin and eosin. $\times 1,000$.

FIG. 23. Section of omentum of Rabbit R 615,—to show a group of monocytes, one of them in division. Hematoxylin and eosin. $\times 1,000$.

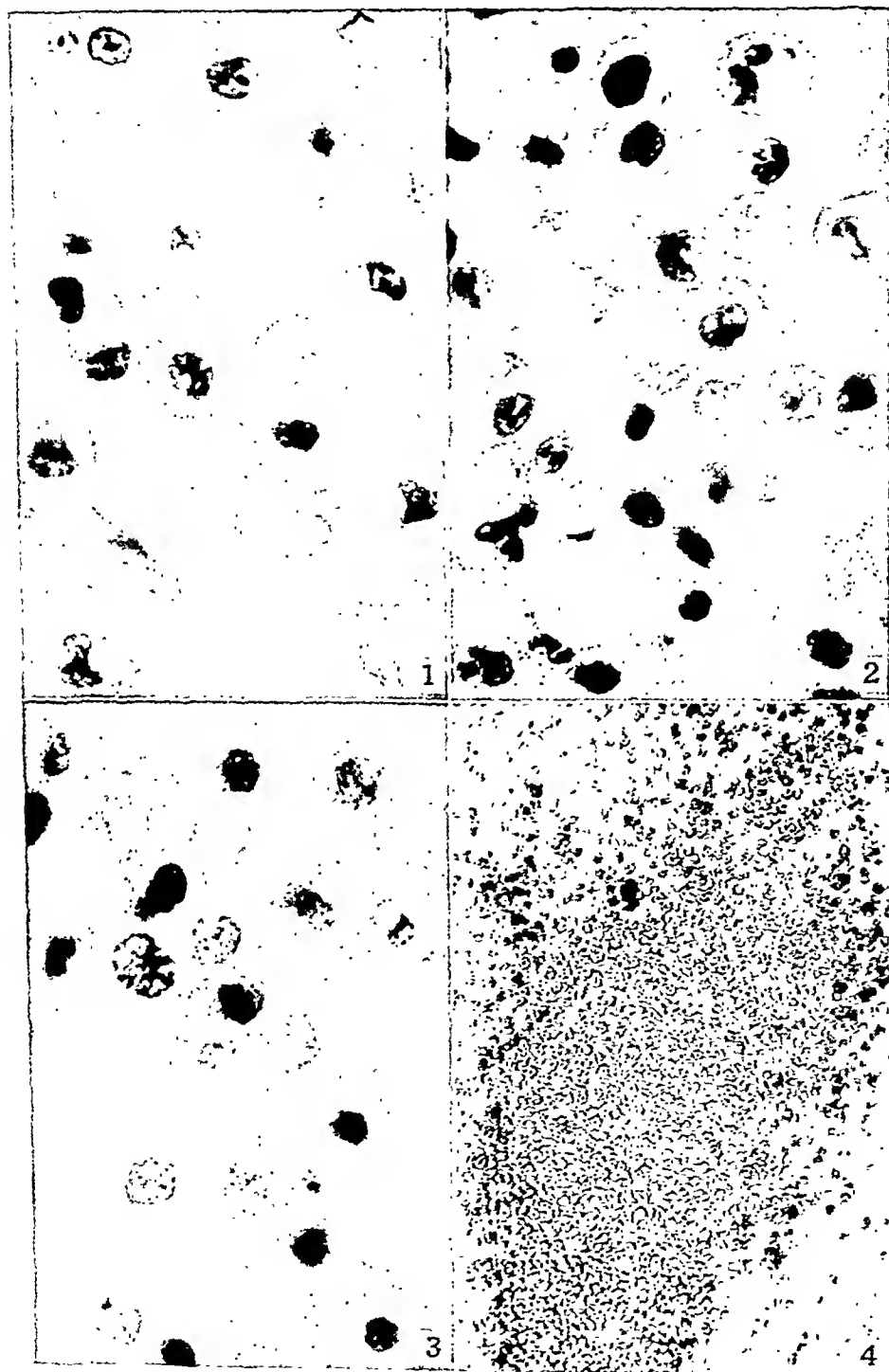
PLATE 7

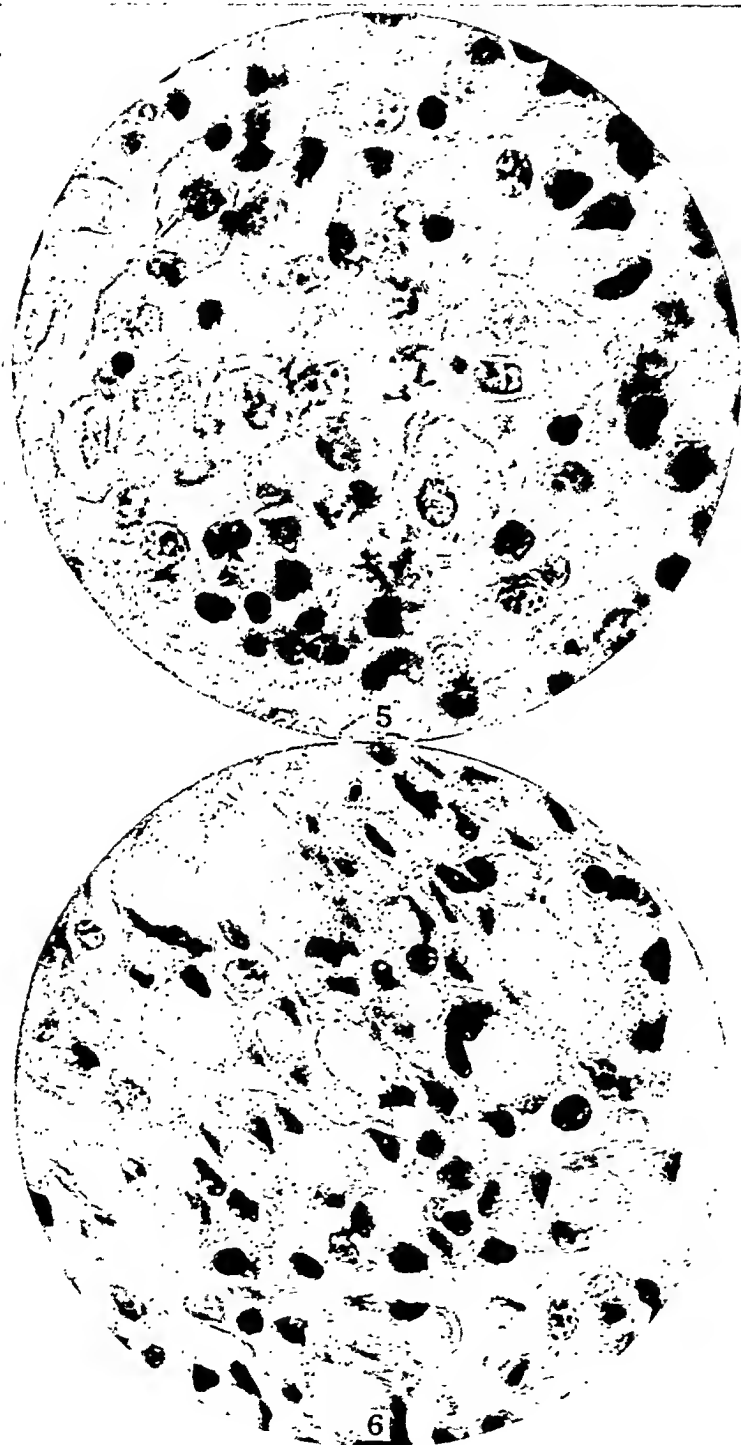
FIG. 24. Section of the increased serosal membrane over the diaphragm of Rabbit R 948, which had received twelve intraperitoneal injections of 16 mg. of soft wax in 0.5 cc. mineral oil. It shows the extreme dilatation and congestion of the small veins and some tubercle-like masses of epithelioid cells diffusely infiltrated with leucocytes. Hematoxylin and eosin. $\times 200$.

FIG. 25. Section of omentum of Rabbit R 618, which had received twelve intraperitoneal injections of 16 mg. of fatty acid III in 0.5 cc. mineral oil. It shows multiple tubercle-like structures with masses of lymphocytes. Hematoxylin and eosin. $\times 200$.

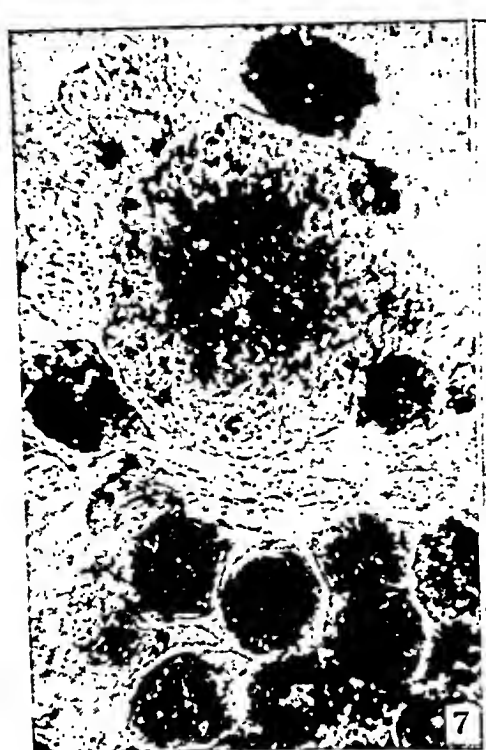
FIG. 26. Section of the mesentery near the mesenteric lymph node of Rabbit R 618. It shows a tubercle-like structure and a giant cell of mixed type which has phagocytized a small cell and shows vacuoles. Hematoxylin and eosin. $\times 200$.

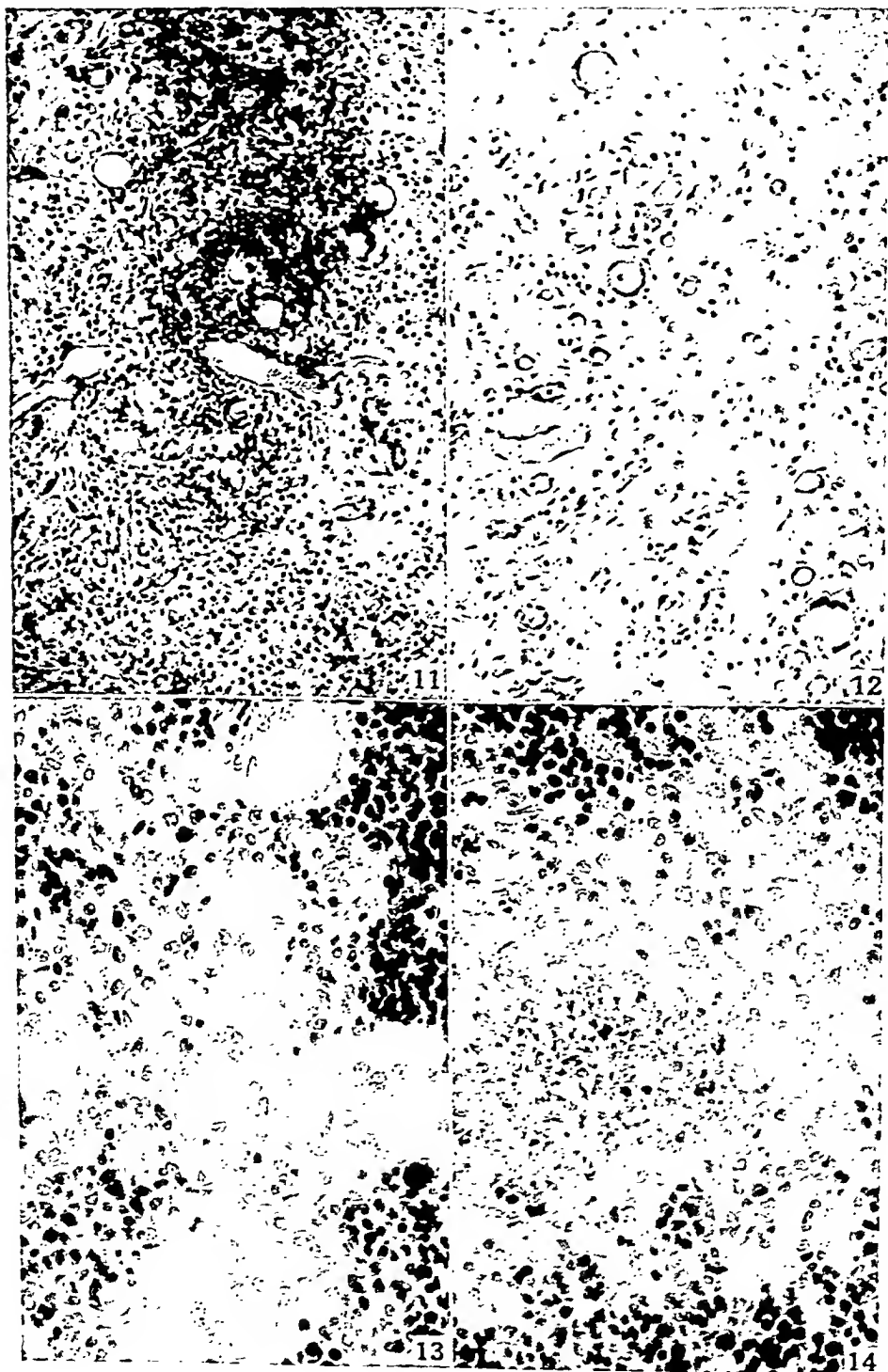
FIG. 27. Section of omentum of Rabbit R 921, which had received twelve intraperitoneal injections of 20 mg. of fatty acid V in 0.5 cc. mineral oil. It shows diffuse epithelioid cells without any arrangement in tubercle-like structures. They are in active division. Hematoxylin and eosin. $\times 1,000$.





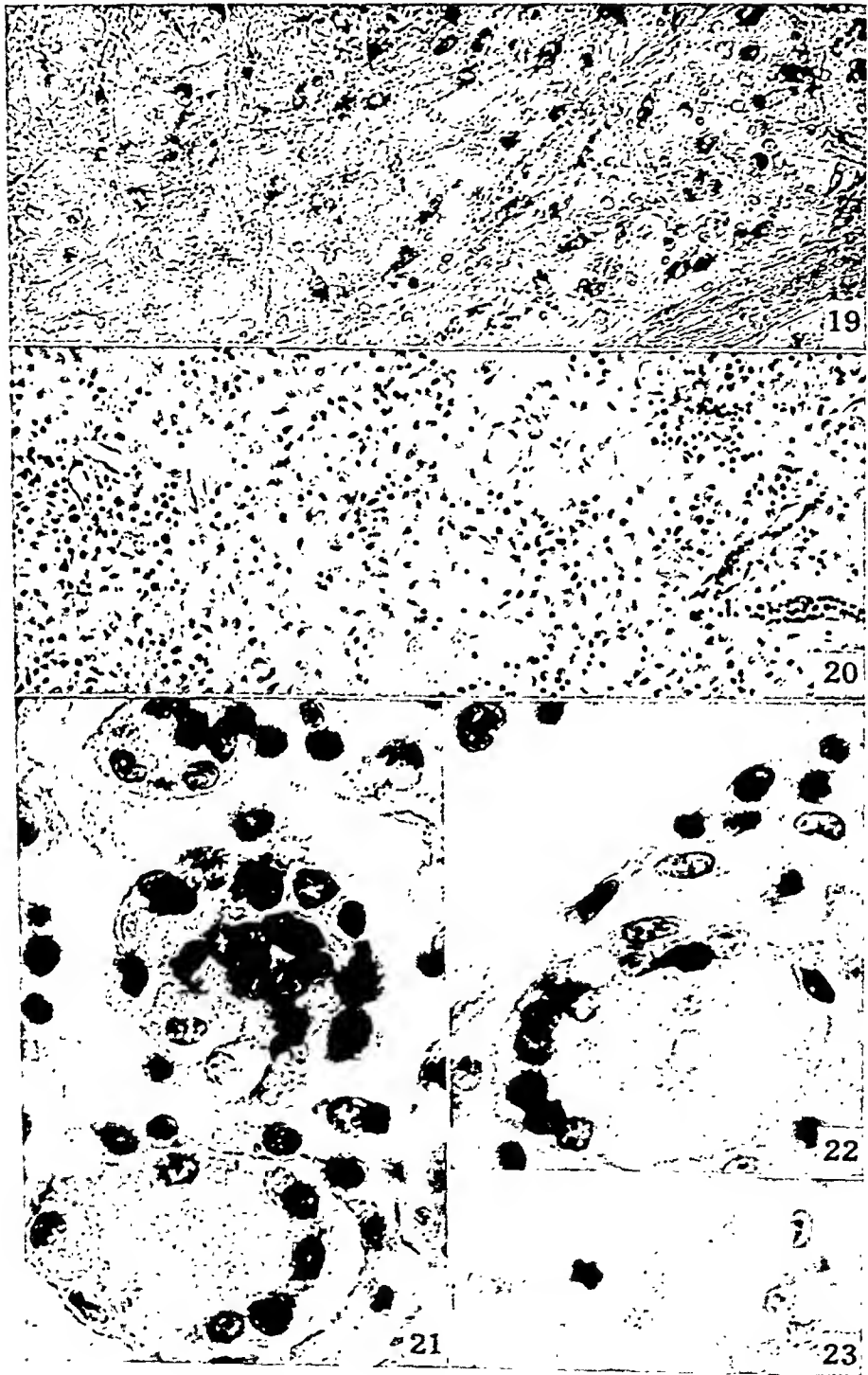
(Sabin, Doan, and Foraker: Lipoids from tubercle bacillus)



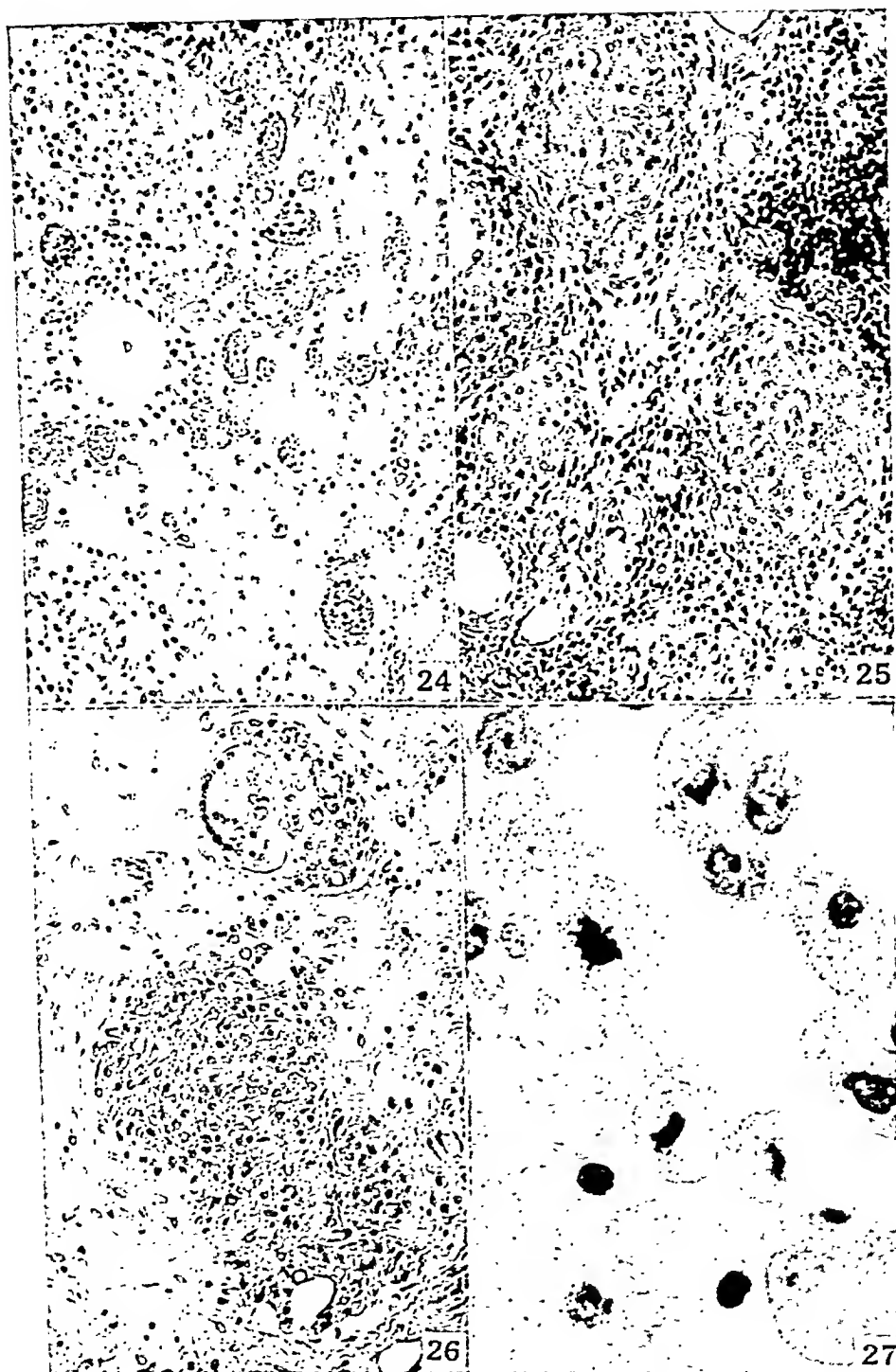




(Sabin, Doan, and Forkner: Lipoids from tubercle bacillus)



(Sabin, Dean, and Forkner: Lipoids from tubercle bacillus)



CHAPTER II

REACTION OF THE CONNECTIVE TISSUES OF THE NORMAL RABBIT TO A WATER-SOLUBLE PROTEIN AND A POLY-SACCHARIDE FROM THE TUBERCLE BACILLUS, STRAIN H-37: SPONTANEOUS PSEUDO-TUBERCULOSIS ASPERGILLINA AS A COMPLICATION IN FRACTION TESTING

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PLATE 8

Reaction to the Protein

In the first chapter (1), we have analyzed the effects on the connective tissue elements of the rabbit, of the intraperitoneal injection of the lipoids from the tubercle bacillus, Strain H-37. With certain of these lipoids, it was possible to reproduce the lesions of tuberculosis in the tissues, including the development of epithelioid cells and epithelioid giant cells in tubercle-like masses and the formation of tubercular granulation tissue. It next became of significance to see if the proteins and polysaccharides of the bacillus, introduced into the tissues by the same route, contributed toward the specific lesions of the disease. We have been able to show that the proteins and polysaccharides thus far tested, when introduced intraperitoneally, do not give rise to tubercular tissue. These substances are soluble in water and hence it is not probable that they remain localized in the subserosal tissues as do the lipoids. Nevertheless it can be demonstrated that they are not inert in their passage through a structure like the omentum.

The intravenous and the intradermal routes are also important for testing the biological reactions to the fractions. From the standpoint of the study of the disease, tuberculosis, the testing of the effects of the proteins and polysaccharides in the tuberculous animal is more important than in the normal.

All these studies are being made under the plan for cooperative research already outlined (1). The analysis of the media in which tubercle bacilli have been grown is being made by Dr. Esmond R. Long and Dr. Florence Seibert at the University of Chicago, who are developing chemical and biological studies of the tuberculo-proteins (2-5). From the media, Dr. Seibert has isolated water-soluble protein in crystalline form; in its basic chemical structure it seems to be the same protein as that obtained from the bacilli by Johnson, Coghill, and Renfrew, and designated 304. Dr. Seibert has shown that this protein is probably the active principle in tuberculin; that it kills sensitized guinea pigs in appropriate doses. Her observations indicate that this protein molecule is extremely labile, readily undergoing changes that affect solubility and biological properties. In its natural form it is soluble and crystallizable; but with slight manipulation the original solubility is so changed that instead of dissolving at pH 5.4 in distilled water it dissolves at a pH of 7.0, and the protein will still give a skin reaction but will not crystallize. A still further manipulation renders it insoluble, except in an alkaline medium, under which conditions the biological properties are lost. Long (6) has made further significant biological studies with this protein. By perfusing the renal vessels of tuberculous swine, he has produced an intra-glomerular tuberculin reaction resulting in a so-called glomerular nephritis.

Under the direction of Dr. Treat B. Johnson, a comprehensive analysis of the chemical constituents composing the tubercle bacillus is being made at Yale University (7-9). After the lipoids had been extracted from the bacilli by Dr. R. J. Anderson, the proteins and other products from the residue have been isolated by Drs. Johnson, Coghill, and Renfrew, who have also followed the increasing development of polysaccharides in the media as the bacilli grow and die in the cultures (10). These polysaccharides have not yet been tested biologically.

The original water-soluble proteins submitted to us for testing were prepared by Drs. Johnson, Coghill, and Brown (7-9), and were designated 304 and 903. The differences in the preparation of these two specimens have been outlined by Dr. Johnson as follows:

"Both 304 and 903 are protein fractions extracted by cold water from unautoclaved tubercle bacilli after defatting for 2 weeks with ether at ordinary temperature.

"The only difference in technic employed is that 304 was precipitated from the cold, sterile, aqueous extract after the latter had been centrifuged in an ordinary high speed cup centrifuge only. No. 903, on the other hand, was precipitated from a corresponding sterile, aqueous extract which had been run through the high speed supercentrifuge twice before precipitation of the protein.

"That 304 was a less pure product chemically was indicated by the results obtained when we analyzed the products for nitrogen.

304	Nitrogen content	14.40 per cent
903	Nitrogen content	15.50 per cent

"I would conclude from our chemical data that 903 probably contains a higher concentration of the pure protein than 304. Also it is very probable that 903 is slightly more soluble, due to its higher purity."

These proteins were tested in 1927 by Sabin and Doan (11), by the intravenous route in normal rabbits.

It was found that though they were far less toxic in the normal than in the tuberculous animal, nevertheless they were not inert in the former when given in sufficient doses. Both specimens were soluble in distilled water and were given from a saturated solution. Protein 903 was the more toxic, the lethal dose being smaller. The effects noted were anorexia, loss of weight, temperatures ranging to 108°F., a rise of leucocytes in the peripheral blood, an anemia, extensive diffuse hemorrhages in organs and connective tissues, marked proliferation of clasmatocytes in the interstices of the septa of the lungs, causing a so-called interstitial pneumonia, and, in sufficient doses, death. These studies indicate that certain toxic phenomena which characterize the infection of tuberculosis can be produced in a normal animal by intravenous injection of the active protein in sufficient dosage.

New samples of water-soluble proteins from the bacilli prepared by Drs. Johnson and Renfrew, and designated 304-A and 304-B, have been received more recently. These proteins were prepared from the residue after the extraction of the alcohol-ether-soluble substance by Dr. Anderson. For protein 304-A, the original bacteria were not autoclaved before the alcohol-ether extraction, while for 304-B they were inactivated. Both of these samples proved to be much less soluble in water than either of the original proteins, 304 and 903. In making up the saturated solution, there was a large insoluble residue; correlated with this was a decreased toxicity (10).

A series of eleven doses of the saturated solution, 10 cc. each, were injected intravenously into four rabbits (R 311 and R 318 receiving 304-A, and R 313 and R 316, 304-B). The animals showed no loss in weight; there was fever to 106°F. and 107°F.; in all but one there was a leucocytosis of 20,000 white cells, the neutrophilic leucocytes being from 10,000 to 12,000 cells. All the rabbits developed a slight anemia with a fall of approximately 1,000,000 red cells, correlated with a drop in hemoglobin of from 1.69 to 3.04 gm. per 100 cc. (Newcomer). There was a tendency toward recovery from the anemia during the injections. At the suggestion of Dr. Johnson we then put a greater amount of protein into solution by first dissolving it in N/10 sodium hydroxide and neutralizing with hydrochloric acid. In this way 5 mg. of the protein were dissolved in 5 cc. of fluid and at this

stronger concentration were given in eight daily doses to Rabbits R 311 and R 313. During these injections there was a second fall in red cells and hemoglobin, of nearly the same magnitude as the first. As in all previous experiments with chemical substances from the tubercle bacillus, intracutaneous tests with Old Tuberculin, the protein, the phosphatide, and the polysaccharide were consistently negative. When given intravenously in six antigenic doses of 25 mg. each to two rabbits, R 702 and R 703, this relatively insoluble protein 304 produced no demonstrable precipitins in the blood serum.

The four animals receiving the daily injections of protein were killed while in excellent condition, and showed in general the same effects, which were not more marked in the rabbits that had received the extra five doses. There were minute hemorrhages into the bone marrow in three and in the lungs in the fourth; the one without hemorrhages in the bone marrow (R 316) had the least anemia. There were small patches of thickened septa in the lungs and in three of the four an increase of leucocytes in the spleen, both free and engulfed in clasmatoocytes. The hemorrhages might account for the anemia, and the leucocytes in the spleen probably reflected the leucocytoses in the peripheral blood, for they were not found in the animal without leucocytosis.

These experiments with different samples of the proteins have shown that there exist considerable variations in potency. The chemical studies now in progress are being especially planned to assure protein fractions of uniform nature and biological activity. While much more toxic to tuberculous animals, the proteins from the bacilli, when not denatured, are also toxic to normal animals when given intravenously in sufficient dosage. The toxicity has consistently paralleled the solubility in water. In general the effects of the proteins with the possible exception of the hemorrhages have been like those of any foreign protein.

In all the experiments involving the intravenous injections of the proteins, no formation of epithelioid cells, either diffuse or in tubercles, has been detected in any of the tissues. There is no proof, however, that substances injected intravenously actually reach the tissues in an unchanged state. Hence we used the intraperitoneal route.

In experiments comparable to those with the lipoids, eight rabbits were given the water-soluble proteins in intraperitoneal doses, four of them receiving protein 304-B from the bacilli, and four 304-F from the filtrate. These proteins were prepared from the human strain H-37 by the H. K. Mulford Company. They were given in distilled water and enough for 3 days was weighed out, dissolved, and kept in the ice box. We now know that on account of the danger of bacterial

growth in the solution, it would have been better to prepare only enough for each day. Protein 304-B was entirely soluble; doses of 20 mg. in 10 cc. water were given daily to four rabbits (R 950, R 951, R 974, and R 975). Rabbit R 950 died 3 hours after the ninth dose. There was no peritonitis but one segment of the cecum was contracted as if traumatized by the needle at the last injection. We were interested to see if there were any signs of a possible slight protein sensitization. The lungs were markedly red and in sections the veins and capillaries were distended with blood; there was an increase in eosinophilic leucocytes in the capillaries of the lungs and a slight increase in eosinophilic myelocytes in the bone marrow. There were marked changes in spleen and bone marrow, both showing an increase in clasmatocytes loaded with yellow pigment; there were congestion in the omentum and bone marrow and slight hemorrhages in the latter. The other three animals received twelve doses. One of them, R 951, had small abscesses in the omentum and a localized peritonitis in the capsule of the liver. Cultures were not taken but Gram stains of the tissues were negative for microorganisms. Two or three typical epithelioid cells were found in the scrapings from the omentum but none could be found in sections. The omentum in all four rabbits in this group showed free leucocytes, clasmatocytes loaded with yellow pigment, and in all but R 950 considerable development of plasma cells. In all, the changes in the bone marrow were marked, consisting in a complete depletion of the fat, considerable depletion of myeloid elements, congestion, hemorrhage, and yellow pigment, both in endothelium and in free clasmatocytes.

Four rabbits (R 956, R 957, R 976, and R 977) received corresponding intraperitoneal injections of protein 304-F. This protein was not completely soluble in water and gave a turbid suspension; there was a small amount of acid-fast debris, a finding entirely lacking in 304-B. Three of this series showed a peritonitis (R 956, R 957, and R 977); cultures were not taken but Gram stains of the tissues were negative for microorganisms in all of them. In Rabbit R 956 there was a thick exudate in which the differential count showed 70 per cent neutrophilic leucocytes (see Table VIII). All four of this group showed a thickening of the omentum with many free leucocytes, some hemorrhage, an increase in new vessels, clasmatocytes loaded with leucocytes or with yellow pigment, some foci of lymphocytes, and a development of plasma cells. Three of the four showed occasional epithelioid cells in the omentum, never in clumps and never enough to suggest tubercular tissue. The bone marrow in all four showed small hemorrhages and an increase of yellow pigment in cells. In two (R 957 and R 977) there was a shift to the left in myeloid elements corresponding to the draining of leucocytes into the peritoneal cavity; in three there was a considerable depletion of fat and myeloid elements; while in the fourth (R 976) there were areas of hyperplasia and zones where the structure of the marrow was quite disorganized by the hemorrhage.

In the entire group of eight animals the two constant signs were free leucocytes in the omentum and hemorrhage. In the four animals in which the free leucocytes were in small abscesses, and infection was suspected, Gram stains were entirely

negative. The marrow showed a depletion of myeloid elements in seven of the eight instances. All the animals showed small hemorrhages in the bone marrow, frequently in the omentum also. In six of the group there was some increase in lymphocytes in the omentum and foci of plasma cells. In the entire group with the exception of the one animal that died the general effects were slight. The fall in the red cells was slight except in one rabbit, R 976, which lost 2,000,000 red cells and 3.2 gm. hemoglobin. All but two lost from 200 to 500 gm. in weight, but this was not significant considering the short duration of the experiment and the fact

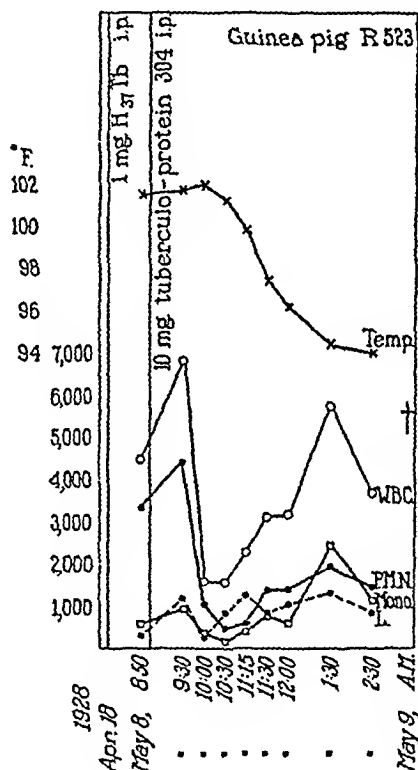


CHART 2

that an adequate base line was not obtained before the injections. The temperature in all reached 103° to 105°F., the upper limits of normal for the rabbit.

The experiment proves hemorrhage to be an effect of the water-soluble tuberculo-protein when administered to normal rabbits. From the standpoint of the general effects of the protein more controls are needed, with cultures and complete bacteriological studies. The experiment demonstrates, however, as a control to the effects of

the lipoids, that the proteins do not produce tubercular tissue by the intraperitoneal route.

In tuberculous guinea pigs (R 523—Chart 2), 10 mg. of the protein 304 given intraperitoneally will cause death within 24 hours, with marked drop in temperature, leucopenia, and showers of monocytes in the peripheral blood.

Reaction to the Polysaccharide

The carbohydrate fraction obtained on analysis of the human tubercle bacillus, Strain H-37, the biological properties of which have been compared with those of the protein and fat fractions, gives strong pentose reactions but its structural formula has not yet been determined. It has been isolated by Dr. Anderson from the small aqueous residue accompanying the original alcohol-ether extract of the bacilli, and it does not include the entire polysaccharide content of the organism. We have studied the action of this particular polysaccharide in normal rabbits, when given by the intravenous and intraperitoneal routes, and in tuberculous guinea pigs on subcutaneous and intraperitoneal injection. This carbohydrate has been designated A-8.

Two rabbits (R 315 and R 317) received 23 daily intravenous injections of 10 mg. of the polysaccharide in 2 cc. sterile glass-distilled water. Daily observations were made of weight, temperature, and blood findings, a preliminary period of 2 weeks serving to establish the normal range in each animal. The rabbits gained just under 1 kilo each during the experiment, with no rise in temperature at any time. The polymorphonuclear leucocytes and monocytes remained within their respective normal limits as did the total white and red blood cell counts and the hemoglobin. The lymphocytes tended in each animal to rise toward the end of the experiment, in one instance from under 2,000 per c.mm. to above 3,000 and in the second from 3,000 to 5,000. Intracutaneous skin tests at two different times, with Old Tuberculin and all three major chemical fractions, were negative and no specific precipitins were demonstrable in the blood serum. The inability of the polysaccharide to elicit demonstrable antibodies as well as its apparently complete inertness when given intravenously was further demonstrated in two rabbits (R 704 and R 705) which received six antigenic doses each of 25 mg. in 5 cc. distilled water over a period of 20 days. No clinical, blood, serological, or skin-sensitive changes were observed. Postmortem studies revealed no gross or microscopic tissue changes that could be detected in any of the rabbits which received the intravenous polysaccharide.

Three rabbits were studied after intraperitoneal injections of the polysaccharide, one (R 454) after three daily injections of 10 mg. in 1 cc. distilled water, and two (R 1022 and R 1023) after twelve injections of 10 mg. in 10 cc. distilled water. With the exception of a transitory fall in total red blood cells in two animals (R 1023 and R 1022), no changes were noted in the number or percentage of any of the cells of the peripheral blood. There was no rise in temperature, and only a slight decrease in weight. It should be noted that the blood counts and the temperatures were taken only once daily.

The tissues of Rabbit R 454 were studied post mortem after three injections for the acute reaction. There was no excess of free fluid in the peritoneal cavity and no evidence of any reaction to the injections except in the omentum. The differential count of the cells in the small amount of peritoneal fluid obtained showed 36 per cent neutrophilic leucocytes, 1 per cent eosinophils, 8 per cent lymphocytes, 43 per cent monocytes, and 12 per cent clasmatoocytes (*cf.* Table VIII). The monocytes were all normal and non-phagocytic, while all the clasmatoocytes were filled with phagocytized red and white blood cells. The omentum in the gross showed an increase in vascularity and in the size and number of the milk spots. Supravivally the most striking point was the large number of highly phagocytic clasmatoocytes, frequently six to eight being found in one oil immersion field, each containing from ten to twelve disintegrating neutrophilic leucocytes and some red blood cells. There were also free granulocytes, some increase in young fibroblasts, and some small clumps of monocytes, but no epithelioid cells. In section, the existence of increased vasodilatation and the predominance of the leucocytic and clasmatoytic reaction were readily confirmed. Much of the tissue remained entirely normal. At one point there was an incipient abscess with a large accumulation of still intact polymorphonuclear leucocytes, and in a few other places very much smaller foci. There were some plasma cells and scattered monocytes but no epithelioids. The mesenteric nodes, spleen, liver, kidneys, adrenals, and lungs were entirely normal in the gross and microscopically. The bone marrow was normal in the gross and showed only a slight depletion of the myeloid elements along the margin.

After twelve intraperitoneal injections of the polysaccharide, the findings (R 1022, R 1023) were much the same as those just described. There was no gross evidence of irritation or reaction on the part of any of the tissues or organs in the peritoneal cavity. The differential counts of the free cells averaged 51 per cent neutrophilic leucocytes, 7.5 per cent lymphocytes, 33 per cent monocytes, 3.5 per cent clasmatoocytes, 5 per cent serosal cells. Figs. 1 and 2, Plate 8, illustrate the appearance of the clasmatoocytes found in the omentum (R 1023), some fusiform and containing finer debris, the majority large and engorged with phagocytized whole blood cells not yet disposed of. Three of these active clasmatoocytes are shown in Fig. 1, and two in Fig. 2. An elongated clasmatoocyte with fine granules is in the lower right corner of Fig. 1, and free leucocytes are shown in both figures. Aside from this finding, together with that of a moderate number of polymorphonuclear cells, there was no evidence of unusual reaction in milk spots or inter-

spaces. This rabbit (R 1023) showed an occasional monocyte with increased rosette of neutral red bodies and one multinucleated cell; the other animal showed only the normal number and distribution of monocytes (Table VIII).

In brief, the only apparent local reaction in normal rabbits to the polysaccharide from the human tubercle bacillus under the conditions stated consists of an accumulation of neutrophilic leucocytes followed by an activity of the clasmotocytes to phagocytize them.

In contrast to these findings in the normal rabbit, it was first noted by White (12) that not infrequently death ensued promptly after the subcutaneous or intraperitoneal injection of small quantities of the tuberculo-polysaccharide A-8 in tuberculous guinea pigs. The animals showed a progressive fall in blood pressure and body temperature from the time of injection to death. Changes in the adrenals were noted post mortem, but the exact explanation of the phenomenon was not wholly clear.

The polysaccharide, when given in the same quantity, 10 mg., to normal guinea pigs, is apparently as innocuous as in normal rabbits. Either no change in temperature ensues, or there may be a fluctuation within 1°F., and the white blood cells during the course of the 6 or 7 hours following injection present a typical non-specific curve (R 425). The neutrophilic leucocytes after a brief preliminary depression rise as the lymphocytes fall, with a reestablishment of the normal relationships within 24 hours. The reaction is the same in character and time relations whether the subcutaneous or intraperitoneal route is chosen.

In Guinea Pig R 425, after a moderate degree of tuberculosis was induced, an intraperitoneal injection of 10 mg. of polysaccharide was followed by a fall of the neutrophilic leucocytes, lymphocytes, and monocytes and a transitory rise in temperature with a maximum of 1.7°. Apparently, however, there is a point reached in the development of the tuberculous infection beyond which the guinea pig becomes highly susceptible to this sugar. Guinea Pig R 493 received 0.01 mg. of virulent H-37 tubercle bacilli on April 5, 1928. Intracutaneous skin tests made on the twenty-third of the same month, with Old Tuberculin and tuberculo-protein 304, showed +++ and + reactions respectively at 48 hours; phosphatide A-3, tuberculo-polysaccharide, and physiological saline were negative. On April 26, 21 days after the inoculation, 10 mg. of tuberculo-polysaccharide were given intraperitoneally and within 5 hours the animal was dead, the fall in temperature and changes in the blood cells being given on Chart 3. At postmortem the animal was

found to have extensive tuberculosis of liver, spleen, omentum, and pancreas. As compared with Guinea Pig R 425 which survived the polysaccharide, there was quantitatively much more extensive tuberculosis in Guinea Pig R 493.

Inasmuch as this fatal reaction to the tuberculo-polysaccharide is so similar to that already mentioned as following the injection of tuberculo-protein 304 (Chart 2), and since it is known that the polysaccharide contains nitrogen, probably in the form of protein, further

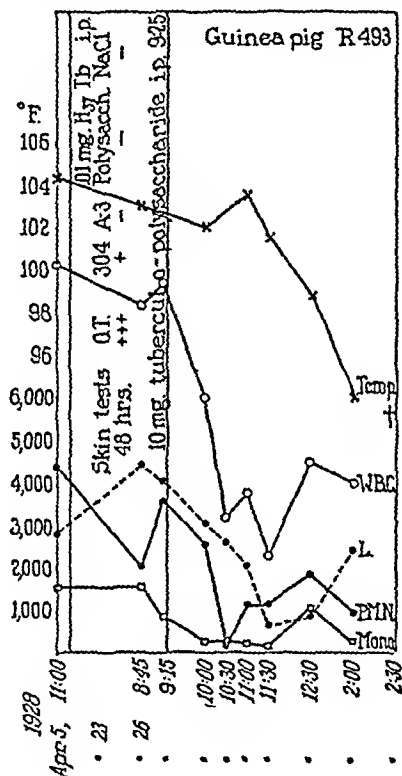


CHART 3

studies are under way to test whether the polysaccharide is toxic *per se* or only as a carrier of protein.

Glucose and pneumococcus type-specific substance III,¹ when given in the same amount to tuberculous guinea pigs as was the tuberculo-protein, caused wholly different effects, the granulocytes tending to

¹ We are deeply indebted to Drs. Avery and Goebel for sufficient of the pneumococcus type-specific polysaccharide for comparative tests with the tuberculo-polysaccharide.

rise within an hour in both instances, the temperature remaining constant after glucose, but rising after the pneumococcus sugar. Chart 4 (Guinea Pig R 498) contrasts two series of observations on the same guinea pig on successive days, the first after pneumococcus polysaccharide and the second after tuberculo-polysaccharide in the same dosage. On the latter occasion it will be noted that after a prolonged precipitous fall in temperature, there was a partial recovery.

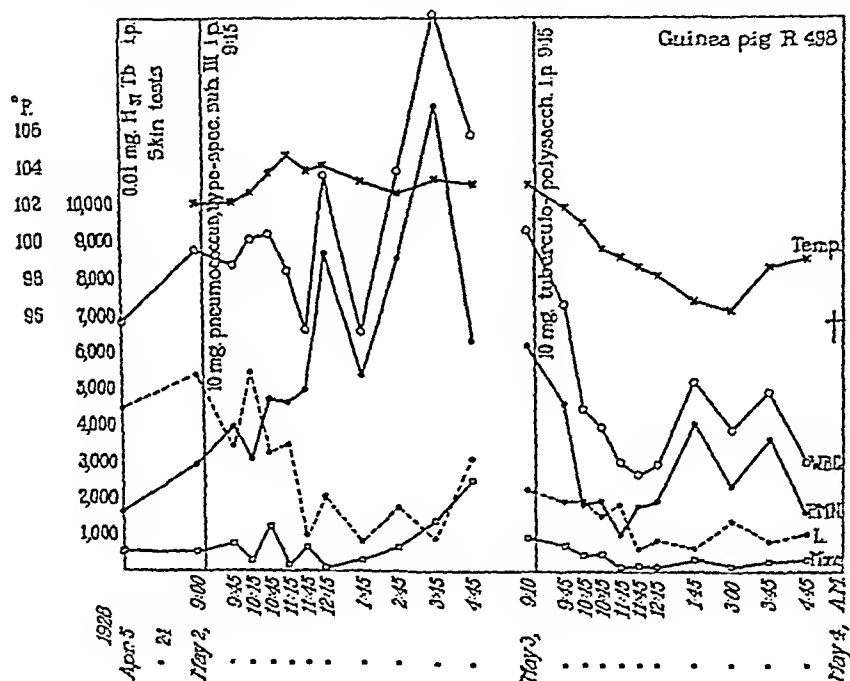


CHART 4

The animal was, however, found dead the following morning. In no instance in which there has been a marked fall in the temperature after the administration of sugar has the animal survived the injection beyond 24 hours. On the other hand, it is our experience that the animal always survives if the temperature remains constant or rises following the injection.

This very marked sensitization of the tuberculous constitution to a specific protein and polysaccharide from the tubercle bacillus seems

to link closely the allergic state of the disease with these soluble substances and tends to show that the phenomenon is not dependent on the lipid fractions.

Pseudo-Tuberculosis Aspergillina

During the progress of the biological testing of the various fractions from the tubercle bacillus in normal stock rabbits, we have on four occasions encountered in the routine autopsies small, translucent, subpleural nodules in otherwise apparently normal lungs. These solitary nodes from 1 to 2 mm. in diameter were indistinguishable in the gross from small tubercles, and inasmuch as fractions from the tubercle bacillus had been given in each instance, it became of immediate importance to ascertain the nature and cause of the lesions.

Two of the rabbits (R 951 and R 975) had received the protein from the bacilli; one (R 945) had been given the unsaponifiable material from the lipoids, and one (R 613) had received fatty acid I from the phosphatide, all the injections having been by the peritoneal route. Microscopically all four of these lesions were found to be similar in nature, under low power appearing superficially not unlike tubercles (Plate 8, Fig. 3). They were small, intra-alveolar collections of epithelioid cells (Fig. 4) and leucocytes, sharply circumscribed by the walls of the air sacs. The alveolar septa were either not involved or only slightly thickened. Supravital studies had confirmed the presence of epithelioid cells in these areas, and mitotic figures were not uncommonly found in these cells in the sectioned material. Polymorphonuclear leucocytes formed a prominent part of the picture in every instance, infiltrating the lesion and being recognizable in all stages of disintegration, both within and without the mononuclear cells. In none of the lesions could acid-fast bacilli be stained. There was found, however, in each original nodule in the lungs, one or more small, encapsulated, fungus-like structures, two of which are indicated by white arrows in Fig. 4, which were identified as pseudo-tuberculosis aspergillina or pneumomycosis aspergillina, as described by Seifried (13).

The distinguishing characteristic, aside from the presence of the fungus, is the intra-alveolar rather than the intra-septal position of the lesion, making a small pneumonic consolidation, sharply delimited and surrounded by entirely normal lung. It becomes of importance, particularly in the testing of fractions from the tubercle bacillus, to be able to recognize these spontaneous tubercle-like lesions in rabbits.

In this connection, Dr. F. H. Heise of Saranac Lake, New York, has called our attention to a case (No. 5110) of human infection with aspergillina under observation for some time at Trudeau Sanatorium, which showed extensive bilateral pulmonary involvement. This patient gave repeated negative reactions to intracutaneous skin tests with Old Tuberculin in all dilutions up to 0.1 mg., which last gave an inflammatory area 6 x 6 mm. Subcutaneous tuberculin up to 10 mg. was repeated twice, and caused no focal or constitutional reaction that could be determined.

Though the cellular reactions to this fungus are so similar to those resulting from infection with the tubercle bacillus that the lesions are spoken of as pseudo-tuberculosis, the allergic state which accompanies the latter is entirely absent in the former, as would be expected. The pseudo-tubercle of the fungus infection, together with the chemically stimulated tubercular tissue produced by the phosphatide fraction from the tubercle bacillus, serves to emphasize the point which we have made elsewhere (14), that the allergic reactions in tuberculosis are not dependent upon the cellular lesion, the tubercle, *per se*, but are associated with the more soluble specific proteins and possibly with the polysaccharides. These observations support from a somewhat different angle the thesis recently advanced by Rich and McCordock (15), that resistance and allergy in tuberculosis need not be considered as identical.

SUMMARY

From these studies we conclude that the water-soluble protein from the tubercle bacillus, when not denatured, is toxic to normal rabbits, inducing fever and hemorrhage when introduced intravenously, but is not lethal except in massive doses. By the intraperitoneal route it is less toxic, but calls forth a local response of leucocytes and phagocytes without any striking proliferation of new connective tissue. A damage to endothelium is indicated by hemorrhage, chiefly in the bone marrow. Tuberculous guinea pigs succumb rapidly to protein 304 when given intraperitoneally.

The polysaccharide is non-toxic when introduced intravenously in the normal animal; introduced intraperitoneally, on the other hand, it is irritative and each succeeding dose continues to elicit a fresh emigration of leucocytes from the vessels. These leucocytes appear to be

damaged, for they are actively engulfed by clasmatoocytes. Guinea pigs with extensive tuberculosis may die soon after subcutaneous or intraperitoneal injections of the polysaccharide.

Aspergillina fungus may produce a pseudo-tuberculous lesion, resembling, both macroscopically and microscopically, the cellular reaction of tuberculosis. The absence of positive skin tests with Old Tuberculin when such lesions are present, as furthermore when tubercular tissue has been produced by chemical stimulation with the tuberculo-phosphatide, emphasizes the necessity for considering tubercle formation as a mechanism apart from allergy in the disease tuberculosis.

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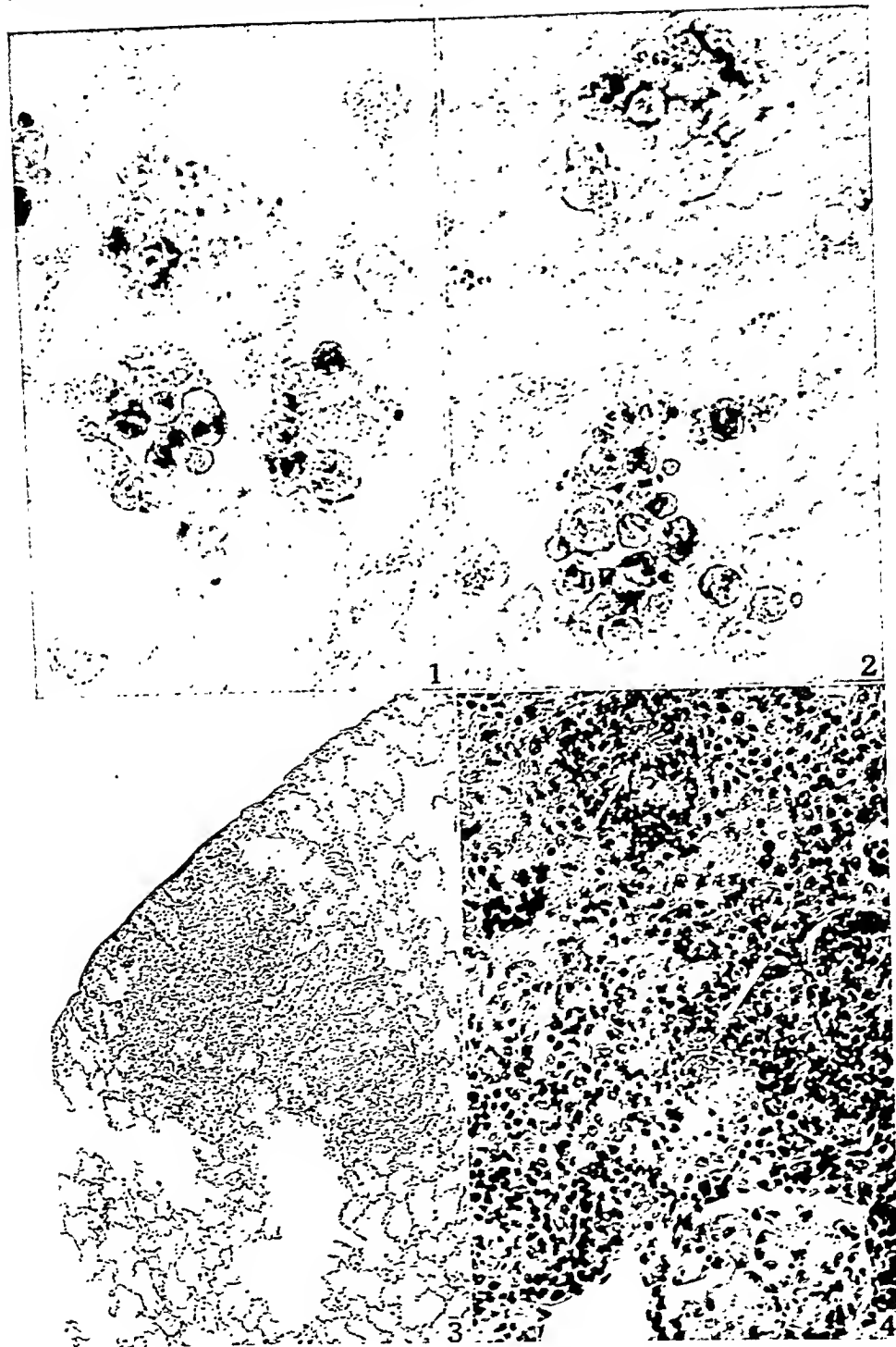
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EXPLANATION OF PLATES

PLATE 8

FIG. 1. Film of omentum of Rabbit R 1023 after twelve intraperitoneal injections of 10 mg. of polysaccharide in 10 cc. distilled water. Photographed while the cells were living, stained in vital neutral red. $\times 1,000$. It shows three clasmatoocytes filled with leucocytes, free leucocytes, and one unstimulated clasmatoocyte in the lower right hand corner. The focus is beneath the serosal cells.

FIG. 2. Film of the same omentum as Fig. 1. In the left hand border is the end of a clasmatoocyte containing heavily stained granular debris.



(Doan, Sabin, and Forkner: Water-soluble protein and polysaccharide)

FIG. 3. Section of the lung of Rabbit R 945 showing a pseudo-tubercle due to the fungus aspergillina. Stained in hematoxylin and eosin. $\times 56$. It shows the intra-alveolar position of the epithelioid cells. The animal had received twelve intraperitoneal injections of the unsaponifiable substance from the purified wax from the tubercle bacilli H-37; the lesion was spontaneous and unrelated to the injections.

FIG. 4. Section of the lung of Rabbit R 975 showing the fungus aspergillina, opposite the two white arrows, and the nature of the intra-alveolar epithelioid cells (lower right hand corner) and free leucocytes. Stained in hematoxylin and eosin. $\times 258$. The animal had received twelve intraperitoneal injections of 20 mg. of protein 304-B from tubercle bacilli H-37; the lesion was spontaneous and unrelated to the injections.

CHAPTER III

THE DERIVATION OF GIANT CELLS WITH ESPECIAL REFERENCE TO THOSE OF TUBERCULOSIS

By CHARLES A. DOAN, M.D., FLORENCE R. SABIN, M.D., AND CLAUDE E.
FORKNER, M.D.

PLATES 9 AND 10

INTRODUCTION

The question of the origin and specificity of giant cells found in the connective tissues under various pathological conditions has been for a long time a controversial one. Their frequency in the lesions of tuberculosis has tended to center many of the discussions concerning the nature and characteristics of these cells about this disease. Consequently, when epithelioid cells and giant cells with peripheral nuclei were found in great numbers as a reaction to the injection of lipoids from the tubercle bacillus (1), it became important to study this type of giant cell which is related to a product of the disintegration of the bacilli and to compare it with the giant cells found in tuberculosis and under other abnormal or pathological conditions.

While the name of Langhans has come to be associated only with the type of giant cell with peripheral nuclei, which is so frequently a characteristic of tuberculous lesions, this author described also the type with scattered nuclei. His studies (2) were made with fresh, teased preparations, so he avoided the pitfalls encountered by subsequent workers who used only sections. Recently Medlar (3) has overcome the difficulty of attempting to judge the distribution of nuclei from one section by reconstructions of selected cells from serial sections. He concludes from his own observations, as does Haythorn (4) after a comprehensive review of the literature, that the available histological criteria do not justify a separate classification of the Langhans type of giant cell with peripheral nuclei from the foreign body type with scattered nuclei. While it is true that many giant cells appear in sections to be transitions between these two types, yet the identification of a rosette of vacuoles as the essential characteristic of the cytoplasm of the monocyte (5, 6) and now of the Langhans type of giant cell as well, may give adequate criteria for such a separation and may open the way to an experimental study of the factors producing giant cells.

The cellular structure of a simple tubercle is well known to be a central core of epithelioid cells with one or more Langhans giant cells, the whole frequently surrounded by a capsule of lymphocytes. Our work in tuberculosis has shown, first, that the epithelioid cell, so abundant in the lesions of this disease, is derived from the monocyte by an accentuation of the rosette of tiny vacuoles; and second, that the epithelioid cell in turn gives rise to the Langhans giant cell by an increase in the number of nuclei. Thus we prefer to designate this type of giant cell an *epithelioid*, or *rosette giant cell*, meaning to indicate that it is merely an epithelioid cell with more than one nucleus.

A considerable variety of epithelioid giant cells are shown on Plate 9, in Figs. 1, 4, 6, 7, 9, and 10. All are characterized by a rosette of vacuoles of an approximately uniform size. In general the vacuoles of the epithelioid type tend to be somewhat smaller than those of the monocyte and they may become as fine and dust-like in the rosette of the giant cell as are those of the cell shown in Fig. 7. There are frequently a few larger vacuoles in the periphery of the rosette as in the monocytes; see for example, Figs. 1, 9, and 10. In the cell of Fig. 1, the vacuoles were demonstrated through the reaction to trypan blue given by repeated intraperitoneal injection, while in all the other cells, the rosette was brought out by the supravital reaction to neutral red. No specific stain visualizes the rosette in fixed tissues, except neutral red as retained by special fixation (7-10), but the zone of the rosette stains faintly acidophilic. In the unstained living cell, the rosette appears as finely granular material around a clear area, the centrosphere. This appearance was recognized by Langhans in his original study.

Considering the rosette as the characteristic structure of this type of giant cell, it will be noted that the nuclei are peripheral to the rosette, making as it were an encasing sphere around it. The ratio of the size of the rosette to the diameter of the cell will determine whether the nuclei are at the periphery of the cell itself, as in Fig. 4, or internal to a wide exoplasm, as in Figs. 1 and 6. The difficulties of determining the type of giant cell in sections can be seen readily by comparing the cells of Figs. 2 and 10. In the case of the epithelioid giant cell of Fig. 10, the nuclei could be made to appear unipolar, circumferential, or scattered according to the plane of section.

There is considerable evidence that the multiple nuclei of the epithelioid giant cells come from amitosis of the nucleus of the epithelioid cell. It is in the monoblasts and the early monocytes that the most frequent mitoses are found, when one studies tuberculous lesions and tubercular tissue produced by lipoids from the tubercle bacillus. Young epithelioid cells also divide by mitosis, as can be seen in the example given in Fig. 5 of the first chapter, Plate 2; but we have never found an epithelioid giant cell showing a mitotic figure. Moreover, in cells such as the one of Plate 9, Fig. 7, which is an epithelioid giant cell from the tibial bone marrow of a rabbit (R 1124) dying from tuberculosis, direct evidence of amitosis is shown. One of the nuclei shows a considerable constriction, another had probably just divided with a second constriction in one of the resulting two, while a third nucleus shows another amitotic fission in progress. These nuclear appearances are identical with those seen in the cells of the blood from a case of subacute, myeloblastic leucemia, described some years ago (11), in which amitotic divisions were followed repeatedly from the beginning constriction to complete separation. It seems to us possible that it is the gradual isolation of the centrosome by the increasing size of the rosette of vacuoles which finally inhibits the process of mitosis in the epithelioid cell and thereby necessitates any increase in nuclei by amitosis.

Associated with the concept that the Langhans giant cell is derived from a single epithelioid cell, is the observation that this type of giant cell has a limited range of size and a limited number of nuclei. Rarely have we found a rosette giant cell with more than twenty nuclei; the cell of Fig. 10 has twenty-two. In contrast, the non-rosette type of giant cell frequently has as many as a hundred or more nuclei. The range of size of the Langhans giant cells is well represented on Plate 9, from the epithelioid cell of Fig. 5, which is 8 to 12 microns in diameter, to the cell of Fig. 9, of about 60 microns. There is no such limit to the size of the foreign body type of giant cell and the one of Fig. 2 is by no means the largest we have seen.

The foreign body types, in contrast to the rosette giant cells, are without cytoplasmic pattern. Forkner (12) has demonstrated that this type of giant cell may be formed by the fusion of cells, most commonly by the fusion of monocytes. For this group, the non-rosette

type formed by fusion of cells, we are retaining the usual name, *foreign body giant cell*, without in any way meaning to signify that the epithelioid giant cell is not also formed in response to the phagocytosis of foreign material, since in tuberculosis it does develop in response to lipoids foreign to the body.

Giant Cells in Tuberculosis

With the identification of the Langhans giant cell as a multinucleated epithelioid cell, it becomes clear that, in so far as the epithelioid cell, either in tubercles or diffusely scattered in the tissues, can be considered as characteristic of tuberculosis, its derivative, the epithelioid giant cell, can also be considered characteristic. Wherever the proliferation of new epithelioid cells occurs in abundance, as in tuberculosis, or in the reaction to tuberculo-phosphatide, giant cells with an identical cytoplasmic structure may appear.

If it is true in tuberculosis that the epithelioid giant cells are simply modified epithelioid cells formed in response to lipoids liberated from disintegrating tubercle bacilli, then this type of giant cell should be the first to appear in the early primary reaction to the infection before there is caseation, or extensive necrosis of tissues, or superimposed secondary infection. When any of these latter conditions exist, the reaction is no longer that of the tissues to the tubercle bacillus or its lipoids alone. Corresponding to the more complex nature of the stimuli, cellular responses of increasing variety occur, and giant cells of much larger size and lacking all pattern in nuclei and cytoplasmic structure appear, together with clasmatocytes and neutrophilic leucocytes. These "foreign body" giant cells we have found in tuberculous areas in direct proportion to the amount of tissue destruction and necrosis present. We have studied the relative incidence of the two types of giant cells in acute fulminating tuberculosis as contrasted with their incidence in the more chronic disease, and in the various organs showing lesions.

In the supravital preparations differential counts of the giant cells have been readily made, and subsequently in the fixed tissues a correlation has also been made of the distribution and location of giant cells with reference to tuberculous foci. Of five rabbits (R 849, R 855, R 877, R 856, R 876) dying within 15 to 30 days after intravenous injections of from 0.5 to 0.75 mg. bovine tubercle bacilli,

Strain B-1, three showed only epithelioid giant cells in scrapings from the lungs, and no foreign body types; the fourth showed only one foreign body (non-rosette) giant cell in several preparations studied from the lung, as contrasted with numerous epithelioid giant cells, and only epithelioid giant cells in spleen and marrow surveys; the fifth, dying at 28 days, while showing a great preponderance of rosette giant cells in the lung, still contained frequent non-rosette giant cells as well.

It is interesting to analyze the fixed tissues in the rabbits which showed this difference in giant cell content in the supravital studies. In the first place, the rosette or epithelioid giant cell is usually present in direct association with epithelioid cells, either in the center, or as an integral part, of a tubercle. In the early stages of tubercle formation in the rabbit, the various phases of the rosette cell from monocyte to multinuclear epithelioid cell dominate the reaction (13).

The giant cells which appear first are few, epithelioid in type, relatively small, and not associated with any visible evidence of tissue destruction. This simple condition may not persist long. When degeneration of epithelioid cells starts within a tubercle with infiltration of leucocytes, or when the growing tubercle causes a pressure necrosis on surrounding tissue or organ cells, both conditions involving a lowering of resistance to secondary bacterial invaders, the stimuli become multiple and the reactions correspondingly more complex. It is at this point that non-rosette giant cells appear, probably in response to larger increments of débris to be phagocytized and disposed of, followed by the more chronic infiltration of lymphocytes, again possibly in the attempt to limit the dissemination of the noxious products of bacterial and tissue destruction. Thus the foreign body giant cells appear relatively later in the course of the disease and are found more frequently at the periphery of the tubercle proper, unless extensive caseation has resulted in their wandering into the area of degeneration in a phagocytic capacity. In the fifth rabbit of the series mentioned above, a somewhat more extensive state of caseation in the pulmonary tubercles, as revealed by a survey of the sections, substantiates, in some degree at least, the relationship of this factor to the increased numbers of non-rosette giant cells in this animal.

In this connection it is significant that the lungs as seen in rabbits experimentally infected with tuberculosis contain, especially in the more chronic stages of the disease, more of the foreign body type of giant cells than any of the other tuberculous organs.

For example, in one survey of this kind (R 1124—killed 62 days after 5,000,000 B-1 organisms intravenously), in which giant cells were being particularly noted, of 25 such cells counted in the supravital preparations from the lungs, 7 were of the foreign body or non-rosette type; the peribronchial nodes and tibial bone marrow showed 4 foreign body to 21 epithelioid giant cells; the spleen, 3 foreign body to 22 rosette giant cells; and the popliteal lymph nodes, humeral and femoral bone marrows showed only the epithelioid type of giant cell. The humerus exhibited 25 epithelioid giant cells to 3 megacaryocytes and no foreign body giant cells. In three rabbits (R 734, R 954, R 955), surviving for from 100 to 196 days, preparations from the lungs, in which chronic tuberculous lesions were present, showed both kinds of giant cells, though the epithelioid type predominated; the kidneys, however, showed in the supravital preparations only the epithelioid type. The tendency to destruction of lung tissue and cavitation in chronic pulmonary tuberculosis probably accounts for the frequency with which different types of giant cells have been encountered in this organ in the disease, and for the conclusion so frequently drawn that there is therefore no basis for attributing significance to any particular kind of giant cell in tuberculosis.

The observations given above with the detail limited to a few selected experiments are representative of the studies carried on in this laboratory during the past 5 years upon much larger numbers of animals, under a wide variety of conditions centering about experimental tuberculosis.

It would seem clear that in tuberculosis the Langhans or rosette giant cell can be considered, together with the epithelioid cell, as a direct reaction to the freeing of certain lipoids, notably the phosphatide and certain fatty acids, from disintegrating tubercle bacilli, while in general the reactions in which foreign body giant cells appear are the result of multiple and much more complex stimuli.

Giant Cells after Tuberculo-Phosphatide

In a preceding chapter (14), we have presented evidence showing that certain lipoids from the tubercle bacillus produce the epithelioid cell and tubercle-like structures characteristic of the disease. It is with the giant cells found in response to these lipid fractions that the interest centers here.

Comparable phosphatide fractions (A-3) from human, bovine, and avian tubercle bacilli have all given the reactions in the tissues characteristic of tuberculosis, that is to say, epithelioid cells distributed diffusely and in clumps resembling tubercles, together with typical

rosette giant cells having peripherally placed nuclei. Repeated supravital surveys of omentalspreads and tissue scrapings from numerous animals have shown a marked predominance of rosette giant cells, with but few foreign body types. Indeed, in these experiments the number of the rosette giant cells has far exceeded anything we have ever seen in the disease itself.

For example, the lymph node illustrated in Fig. 13, Plate 4 (14), contained what might be called an almost pure culture of Langhans' giant cells. The extreme number of giant cells found in these reactions with the fractions is probably due to the fact that the amount of tuberculo-lipoid concentrated in the tissues is overwhelmingly greater than that ever liberated in the disease in the same period of time at any one focus.

The cells of Figs. 4 and 5 have been drawn from a supravitaly stained spread of the omentum of Rabbit R 684 after intraperitoneal injections of avian phosphatide. It will be seen that the multinucleated cell (Fig. 4) has a massive, unified, central rosette, an accentuation of that seen in the epithelioid cell of Fig. 5, in contrast to the foreign body giant cells of Figs. 2 and 3. The nuclei are far out toward the periphery of the cell, presumably crowded out to this position by the material constituting the rosette. Many of the nuclei have conspicuous nucleoli. This cell is identical with the epithelioid giant cells found after the administration of the other phosphatides, and in tuberculous processes. The epithelioid cell (Fig. 5) is likewise typical of those found in tuberculosis. The fixed sections of the tissues from this animal, as in the others receiving phospholipin, demonstrated the presence and domination of these cell types, in arrangement closely simulating tuberculous tissue.

When the phosphatide was given intraperitoneally in large doses, the reaction in the tissues could not be considered as exclusively due to the phosphatide, because leucocytes were always called from the vessels by the first injection and were phagocytized and destroyed. In a series of experiments to be reported subsequently in greater detail (15), the phosphatide was given intravenously in small antigenic doses and some of the animals used demonstrated the specific reaction to the phosphatide entirely uncomplicated by any other processes. In these animals there were small, scattered foci of epithelioid cells and rosette giant cells, or single epithelioid giant cells in an otherwise entirely normal lung, lymph node, or spleen. This material furnishes the most unequivocal evidence of the direct specific relation of the epithelioid cell and the corresponding giant cell to the phosphatide as stimulus. A single giant cell from the lung of one of these intravenously treated animals is reproduced in Fig. 9, Plate 9. The rabbit (R 844) had received three doses of 10 mg. each of the phosphatide A-3 from human strain H-37 at 5 day intervals. In this giant cell it will be seen that one nucleus lies within the mass of neutral red staining bodies, the remainder of the nuclei being peripherally placed. This is explainable upon the three-dimensional aspect of

the unsectioned spherical cell; the less dense area in the center of the cell indicates the presence of a centrosome about which the neutral red bodies group characteristically in the rosette cells. The presence of ingested carbon particles within the larger vacuoles found so commonly in the phagocytic cells of the lungs helps to identify the organ source of this particular cell and also emphasizes the point that both macrophages or clasmotocytes and rosette cells of the monocyte strain may contain these carbon particles. Nucleoli were very prominent in the majority of the nuclei in this cell. No typical foreign body, non-rosette, giant cells were found.

From the foregoing it will be seen that the phosphatide from the tubercle bacillus introduced into the tissues produces a marked predominance of epithelioid cells and epithelioid giant cells with a minimal number of foreign body giant cells, in this respect closely approximating early non-caseous tuberculosis; while introduced in small doses intravenously, it may give a pure reaction of mono- and multinucleated epithelioid cells.

Giant Cells from the Other Lipoid Fractions from Tubercle Bacilli

The formation of giant cells following the injection of the other lipid fractions accompanied a more complex reaction in the tissues. Either the formation of epithelioid cells predominated, in which case the rosette giant cell likewise predominated; or there was about an equal distribution between the two types of giant cells and fewer epithelioids; or the non-rosette type was in greater numbers. The chemical analysis of the lipid is outlined in Table I of the first chapter (14). There it will be seen that certain saturated fatty acids were isolated from each of the four original fractions and carried the specific tendency toward the formation of the epithelioid cell. This was only true when these fatty acids were finely dispersed in mineral oil, for when introduced undiluted, they were corrosive in action.

In the case of the fatty acid from the phosphatide, all the giant cells seen in the fresh preparations were small and of the rosette type; in sections of this material, however, only about half of the giant cells have the typical nuclear arrangement of the Langhans type, while the remainder are like the giant cells shown in the upper part of Fig. 16 and the lower part of Fig. 17 on Plate 5 of the first chapter (14). That is to say, they show nuclei in a dense mass, rather than in the periphery of the cell. From the supravital studies it seems likely that this appearance is due to the plane of section and that these giant cells were for the most part of the rosette type.

In the analysis of the fatty acids, Dr. Anderson fractionated those from the glycerides into two components, tuberculo-stearic acid and phthioic acid.

The tuberculo-stearic acid was followed by the production of the two types of giant cells in about equal numbers and the phthioic acid induced predominantly the rosette types (Fig. 6, Plate 9). Fig. 6 is a cell taken from the omentum (R 918) after intraperitoneal injections of fatty acid IV and illustrates some of the variations which are often found in these cells. Refractive, even sized, unstained vacuoles are scattered in the cytoplasm at the periphery of the cell. The nuclei are not arranged quite so definitely at the periphery of the rosette, three of them being shown underneath and apparently invading to some extent the dense central pattern of neutral red bodies.

Fig. 10 represents one of these rosette giant cells taken from the omentum of Rabbit R 921 which received fatty acid V. It is one of the largest of the epithelioid type we have encountered, containing 22 nuclei, asymmetrical in size and arrangement, surrounding a rosette of neutral red staining bodies smaller in circumference than is commonly found in a cell of this size. The nuclei, which almost all contain prominent single nucleoli, are arranged in single, double, or triple layers closely surrounding the rosette. The periphery of this rosette shows larger vacuoles with and without the stain, a common finding in the rosette cells.

The chemical analyses gave an opportunity to study the reactions of the tissues to a large molecule and then to its component parts. An interesting example of this is the effect of the purified wax and its two components, the unsaponifiable substance and fatty acid II.

Differential counts of the giant cells after administration of the purified wax (in R 937) showed ten epithelioid giant cells to every one of the foreign body type. This tendency was carried directly by fatty acid II which gave rise to the giant cells, on Plate 6. They were especially typical of those seen in the infection. The unsaponifiable material (in R 945), on the other hand, produced as much new tissue as the phosphatide, but this was non-specific in type, and the counts of the giant cells were the reverse of the above, namely, ten to one in favor of the foreign body type. The same relationship of giant cell types was found with the soft wax (in R 948). After both of these materials, the serosal cells were markedly irritated and there were many highly phagocytic clasmatoctes in the omentum.

Oleic and palmitic acids were found in the phosphatide molecule and, as already mentioned, an isomer of stearic acid in the glycerides. Of these three fatty acids, oleic acid proved on biological testing (R 1024, R 1025—twelve injections of 0.1 cc. each) to be the most irritating to the tissues of the peritoneal cavity. Neutrophilic leucocytes, highly phagocytic clasmatoctes, and serosal cell irritation dominated the reaction; few monocytes and no epithelioid cells were found. Only

occasional foreign body giant cells were present. Palmitic acid also proved to be primarily an irritant with an increased activity of the clasmatoocytes. Few monocytes were found and only one small rosette multinucleated cell was seen in two animals studied (R 1026, R 1028—twelve injections of 16 mg. each in mineral oil). Stearic acid, however, produced upon the injection of from 60 to 240 mg., in divided doses in mineral oil (R 1057, R 1058, R 1059), a minimal but appreciable reaction of monocytes with occasional epithelioid cells and small epithelioid giant cells. A few neutrophilic leucocytes, a moderate number of clasmatoocytes, and an occasional foreign body giant cell completed the cellular detail of the moderate reaction to this fatty acid. There was a marked difference in the size of individual giant cells, those with a central rosette being very much smaller than the ones without this definite cytoplasmic pattern.

Glycerol was also used as a control for these tuberculo-lipoids. When it was given in doses comparable to those of the fatty acids, the reaction (in R 640) was found to be mild, but with epithelioid cells and a few small accompanying rosette giant cells present.

From this study of the tuberculo-lipoids, it is clear that there are certain fractions, notably the phosphatide and certain fatty acids, which give a direct stimulus to the formation of epithelioid giant cells. It is our opinion that this reaction is due to the phagocytosis of the lipid. On the other hand, there are certain partitions of the lipid, notably the unsaponifiable material and the soft wax, which produce predominantly foreign body giant cells. As yet we have no crucial experiment, such as the one with small intravenous injections of the phosphatide, to analyze whether these foreign body giant cells have been produced as a direct response to these fractions, or indirectly through tissue irritation.

Reaction to Different Menstrua Used for Introducing Substances into the Peritoneal Cavity

We have never found multinucleated cells in the normal omentum (14). When distilled water (in R 709) or physiological salt solution (in R 730) is repeatedly injected into the peritoneal cavity of normal rabbits no evidence of irritation or cellular stimulation is seen on casual inspection. Microscopically a slight increase of phagocytic activity on the part of the clasmatoocytes of the inter-milk spot areas may be noted, and among the monocytes normally present in milk spots there is an occasional epithelioid cell, mono- or binuclear. No giant cells of any type, however, are present. Consequently, these two

menstrua are the diluents of choice for the carrying of water-soluble substances into the tissues for an assay of their biological activity.

In the case of the fatty acid fractions and certain other of the lipid fractions from the tubercle bacillus, it has, however, been necessary to use other vehicles. In the search for a bland non-irritating oil with which to dilute the more irritating corrosive fatty acids we have tested intraperitoneally olive and mineral oils among others. The reaction to repeated injections of olive oil has varied somewhat with the individual animal.

Olive oil has given a marked irritating effect, but the cellular response has ranged from a predominant reaction of clasmotocytes and neutrophilic leucocytes with numerous large foreign body giant cells (R 338, R 341) at the one extreme, to a mixed reaction in which monocytes, epithelioid cells, and rosette giant cells equalled or exceeded in number the cells comprising the less characteristic reaction (R 665, R 829; see protocols, page 57).

In Rabbit R 665 (see protocol, page 57) both types of giant cells were abundant; the foreign body type was very large with frequently more than 100 nuclei in a single cell; the epithelioid giant cells were relatively small with less than 20 peripherally placed nuclei. Careful comparative study failed to reveal any distinguishing differences in the size or character of the nuclei in the two types of giant cells which were often present in the same oil immersion field of the microscope. Monocytes with rosettes of normal size and character were numerous, and epithelioid cells were relatively fewer.

A comparative study of the character of the cytoplasmic bodies appearing in the two types of giant cells was provided through the mixed character of the cellular response to olive oil. Rabbits were injected at the same time with trypan blue by various routes and the cellular reaction to olive oil plus this vital dye was studied in unstained and supravitaly stained preparations.

For example, Rabbit R 940 received nine daily intraperitoneal injections of 5 cc. each of olive oil, and during the same period by the same route seven injections, each of 4 cc. of a 1 per cent aqueous trypan blue solution, at 48 hour intervals. The animal was autopsied 12 days after the beginning of the experiment, at which time it was intensely blue, more especially in the tissues of the peritoneal cavity. The omentum showed considerable thickening at autopsy. On microscopic examination a great many giant cells were seen. Over half of the multinucleated cells contained unified central rosettes of fine trypan blue bodies with peripheral nuclei as illustrated in Fig. 1, while the remainder had scattered nuclei and larger segregation vacuoles similar to those seen in Fig. 2.

It is evident that the trypan blue in the epithelioid giant cell has been segregated into the "finely granular" apparatus, which was described originally by Langhans as a characteristic of the unstained epithelioid cells, and which the neutral red only makes more apparent as a typical cytoplasmic rosette. The trypan blue here makes prominent the apparatus of the cell in the same manner. When such cells as that shown in Fig. 1 were subjected to neutral red, introduced so that the process of staining could be watched with the oil immersion lens as it occurred, only the bodies already showing the trypan blue became purple and then violet as the neutral red superimposed itself upon the original dye. This phenomenon as just described for the rosette giant cells, applied in every particular to the reactions observed in the epithelioid cells of the same preparations, many of which contained the dye. In sharp contrast to the finely divided state of the trypan blue as found in the group of cells just mentioned, the numerous clasmatoocytes or macrophages of the omentum were heavily laden with large, coarse, irregular aggregations of the dye, which had no typical pattern to their distribution in the cytoplasm. The large foreign body giant cells, likewise, showed scattered accumulations of blue dye, some of it finely divided but much of it in larger flocculations. There was evidence in some of the foreign body giant cells of an apparent fusion of mononuclear cells, identified in many instances as monocytes by individual rosettes still adjacent to their original nuclei. These observations lend support to the theory that foreign body giant cells are the product of the fusion of mononuclear cells many of which are of the monocyte type. The individual rosettes instead of fusing into one large central rosette to make an epithelioid giant cell lose their identity shortly after joining with others and the nuclei assume positions without an evident pattern within the larger cell. The bone marrow and lymph nodes of this animal showed only clasmatoocytes (macrophages) taking up the trypan blue. The developing monocytes in the peripheral lymph nodes contained none of the trypan blue. This is in conformity with the observation that clasmatoocytes and foreign body giant cells take up the dye much more readily than do the monocytes or epithelioid cells, which must be subjected to repeated direct contact with large quantities of the dye before showing the type of reaction just described, and shown in Fig. 1. The fixed sections

confirmed the findings disclosed by the supravital studies. Sections prepared and stained with Giemsa illustrated the unified central area of the epithelioid giant cells, whereas the cytoplasm of the foreign body giant cells gave evidence pointing toward their development as a result of the fusion of individual mononuclear cells, monocytes, and macrophages.

With mineral oil (Nujol), rabbits (R 660, R 661, R 662, R 663, R 1030, see protocols, pages 58, 59) varied in the amount of reaction to a given dosage as had those treated with olive oil (14); there was, however, never as much irritation with the former as with the latter.

Usually the peritoneal cavity showed little macroscopic evidence of reaction, the omentum having only a slight increase in number and size of milk spots. Both monocytes and clasmatoocytes with occasional giant cells of both types represented the mild increase of cells found in the omentum. Most of the cells were filled with droplets of the injected oil, but there was little evidence of other embarrassment of the tissue in coping with the foreign material. In one animal (R 661) the predominant reaction was of clasmatoocytes and fibroblasts with three giant cells of foreign body type to every epithelioid giant cell. No intermediate or equivocal types of giant cells were encountered. In going over one entire preparation from another rabbit (R 662), ten rosette cells with three or more nuclei were found to two foreign body types. In still another (R 663), six rosette giant cells containing from three to six nuclei were seen in covering some forty microscopic fields, and a like number of foreign body giant cells were encountered.

In general, the reaction to mineral oil was a mild, mixed one, clasmatoocytes vying with monocytes for dominance, and these being accompanied by undifferentiated cells, and by giant cells of both types, containing oil droplets.

Giant Cell Reactions to Typical Foreign Bodies

To complete this survey of giant cell specificity, we included a study of the reactions, as analyzed by supravital stains, to three substances typically representative of the foreign body type, agar-agar, aleuronat, and lycopodium spores. The first material, while relatively resistant, can be eventually handled and probably completely absorbed and removed by the body cells. The second has in the past been used primarily to elicit a response of neutrophilic leucocytes through its property of sterile, chemical irritation. The third is a non-absorbable, foreign spore against which the tissues protect themselves without

elimination of the irritant. The relative sizes of the particles making up the two last mentioned substances may have influenced to some extent the differences observed in the respective reactions.

When sterile, warm, plain agar at pH 7.4 in a state that is just fluid is introduced in small amounts into the subcutaneous tissues it soon becomes well walled off with connective tissue. 7 days after the subcutaneous injection of agar into the abdominal wall at four points, and into the right groin, of Rabbit R 646, a biopsy (with ether) was performed and one of the abdominal areas excised for study. A scraping from the tissue about the agar showed that a great majority of the cells possessed granules or vacuoles of irregular size and shape, which were not distributed in any uniform pattern in the cytoplasm. Only a small percentage of the stained cells appeared to be monocytes though there were a few epithelioid types. Very few polymorphonuclear neutrophils and no lymphocytes were seen. Among the many foreign body giant cells were a considerable number of unstained cells of irregular shape, some branched and some with multiple nuclei. The character of these cells was not clear, and whether they were the precursors of the foreign body giant cells could not be determined from this tissue. The fixed sections of the tissue showed the breaking up of the agar by the invasion of strands of cells, most of which were in the form of large, foreign body giant cells, apparently attempting to surround and isolate smaller masses of agar.

7 days after the biopsy and 14 days after the agar injections the animal was killed and autopsied. The tissue from the inguinal region showed many mononuclear cells with hazy, amorphous granules or vacuoles such as had been seen in the biopsy material. There were many giant cells of the foreign body type with even sized nuclei scattered through the cytoplasm. Among the nuclei were many bodies both stained and unstained by the supravital dyes. A considerable number of clasmatocytes were present and many oil immersion fields contained from two to ten monocytes. The tissues from the subcutaneous areas on the abdominal wall were quite similar except for the presence of fewer monocytes.

Rabbit R 644 was injected intraperitoneally with 11 cc. of sterile, plain agar and in the abdominal musculature with 5 cc. 14 days later the animal was killed by the intravenous injection of air. A mass of sharply circumscribed agar was found in the abdominal musculature without evidence of reaction other than the connective tissue capsule immediately surrounding it. In the abdominal cavity were several small pieces of free agar, in addition to those embedded in the omentum. The latter organ was considerably thickened and moderately injected, but no general peritoneal irritation was present. The spleen was about twice normal size and on its surface were a few translucent nodules. No other gross abnormalities were detected.

Microscopic examination of the living cells around the agar in the abdominal wall showed the presence of many monocytes. Some typical epithelioid cells were encountered, a few with two, three, or more nuclei placed peripherally about

the central rosette. There were, however, many more of the foreign body giant cells found as a part of this reaction, the differential count revealing twenty-one of this type as contrasted with eight of the rosette or epithelioid type, the former being in each instance much larger than the latter when individual cells were compared. While the two types were usually not to be confused, as judged by the differential criteria previously given, occasional cells were found which did not belong clearly to either type.

The predominating cell in the scraping from the omentum was the monocyte. A few were seen with accentuated rosettes, the so-called epithelioid cells. Typical epithelioid giant cells were present, but they rarely contained more than four or five nuclei. On the other hand, there were a great many foreign body giant cells, most of which contained many nuclei, and much neutral red staining débris. Fig. 3 represents a cell taken from the omentum of this rabbit and illustrates the similarity which exists between this type of giant cell and those found secondary to other irritants, Figs. 2 and 8. The milk spots in a spread of the intact omentum were somewhat increased in size, but apparently not increased in number. Some of the milk spots were made up predominantly of clasmatoocytes and others of monocytes. Both epithelioid giant cells and foreign body giant cells were present in some of the milk spots.

This experiment, as well as many others of similar nature, has convinced us that when bland substances are introduced, giant cells of either of the types under consideration may be found without necrosis. Under pathological conditions it is probable that foreign body giant cells are only formed secondarily to areas of necrosis or caseation.

A differential count of the peritoneal fluid from the animal under discussion showed 74 per cent monocytes, 15 per cent clasmatoocytes, 3.5 per cent lymphocytes, 3.5 per cent neutrophils, 3 per cent serosal cells, and 1 per cent unclassified cells with a total count of 5,600.

A study of the fixed tissue in section showed again the agar infiltrated with cells and in the process of being broken up into fragments of various size and shape. Many of the smaller fragments were completely surrounded by giant cells, which were for the most part very large, with nuclei scattered without pattern through the cytoplasm. The areas of the omentum which were free of agar were entirely normal and uninvolved in the local reaction. The other abdominal organs showed no significant changes from the normal.

The recognition in these preliminary studies of a phase of monocyte stimulation in the general tissue reaction to agar suggested to one of us (12) the possibility of producing, if sufficient foci could be established, a monocytosis in the peripheral blood similar to that observed in tuberculosis.